Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 140 (2006) 1–34
veterinary parasitology
www.elsevier.com/locate/vetpar

Review

Diagnosis of bovine neosporosis

J.P. Dubey [a,*], G. Schares [b]

[a] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agricultural, Beltsville, MD 20705, USA*
[b] *Institute of Epidemiology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Seestrasse 55, D-16868, Wusterhausen, Germany*

Received 19 January 2006; received in revised form 17 March 2006; accepted 22 March 2006

Abstract

The protozoan parasite *Neospora caninum* is a major cause of abortion in cattle. The diagnosis of neosporosis-associated mortality and abortion in cattle is difficult. In the present paper we review histologic, serologic, immunohistochemical, and molecular methods for dignosis of bovine neosporosis. Although not a routine method of diagnosis, methods to isolate viable *N. caninum* from bovine tissues are also reviewed.
© 2006 Elsevier B.V. All rights reserved.

Keywords: *Neospora caninum*; Neosporosis; Cattle; Abortion; Serologic tests; Histologic; PCR; Diagnosis

Contents

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

0304-4017/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2006.03.035

1. Introduction, general biology, and clinical signs

The protozoan parasite *Neospora caninum* is a major cause of abortion in cattle worldwide (Dubey, 2003). *N. caninum* causes abortion in both dairy and beef cattle. Cows of any age may abort from 3 months gestation to term with most abortions occurring at 5–6 months gestation. Fetuses may die in utero, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected. Neosporosis-induced abortions occur year-round (Anderson et al., 1991; Thurmond et al., 1995; Wouda et al., 1998a). There is no evidence that incidence of neosporosis has changed over a decade. Both dairy and beef cattle with antibodies to *N. caninum* (seropositive) are more likely to abort than seronegative cows (Davison et al., 1999d; De Meerschman et al., 2000; Moen et al., 1998; Moore et al., 2003; Thurmond and Hietala, 1997; Waldner, 2005; Wouda et al., 1998a) and up to 95% of live born calves from seropositive dams will be congenitally infected and clinically normal (e.g. Paré et al., 1996). The age of dam, lactation number, and history of abortion have been reported not to affect the rate of congenital infection (Paré et al., 1996) although this conclusion is contradicted by other reports in which vertical transmission was shown to be more efficient in younger than older cows (Dijkstra et al., 2003; Thurmond and Hietala, 1997; Wouda et al., 1998a).

Neosporosis-associated abortion problems in bovine herds may have an epidemic or endemic pattern. There is evidence from epidemiological studies that epidemic *N. caninum*-associated abortions are caused by post-natal infection of naïve cattle most likely via the exposure to oocyst contaminated feed or water (Jenkins et al., 2000; McAllister et al., 2000; Sager et al., 2005; Schares et al., 2002). In some epidemic herd outbreaks as many as 57% of pregnant dairy cows have been reported to abort over just a few weeks up to months (Jenkins et al., 2000; McAllister et al., 1996a, 2000; Moen et al., 1998; Schares et al., 2002; Thornton et al., 1994; Wouda et al., 1999; Yaeger et al., 1994). Abortion outbreaks have been defined as epidemic if more than 10%, 12.5% or 15% of cows at risk abort within 4, 6 or 8 weeks (Moen et al., 1998; Schares et al., 2002; Wouda et al., 1999). Epidemiologic data indicate evidence for protective immunity to *N. caninum*-associated abortion when chronically infected dams are re-infected horizontally (McAllister et al., 2000). This was confirmed when naturally infected dams received an experimental challenge infection (Williams et al., 2003). Fetuses of dams, experimentally infected prior to gestation, were also protected against an exogenous transplacental infection (Innes et al., 2001).

In cattle herds with endemic abortion due to neosporosis there is often a positive association between the serostatus of mothers and daughters, i.e. there is evidence that the major route of transmission in these herds is vertical (Schares et al., 2002; Thurmond et al., 1997). There is evidence that the recrudescence of latent infection during gestation is responsible for an increased abortion risk (Guy et al., 2001; Paré et al., 1997; Stenlund et al., 1999). Several studies demonstrate that chronically infected seropositive cows have an about two- to three-fold increased risk of abortion compared to seronegative dams (Paré et al., 1997; Pfeiffer et al., 2002; Wouda et al., 1998a). Thurmond and Hietala (1997) observed a 7.4-fold higher risk of abortion during the first gestation of congenitally infected heifers. A small proportion (<5%) of cows may have repeated abortion due to neosporosis (Anderson et al., 1995).

Clinical signs, other than abortion, which have only been reported in calves <4 months of age, include neurologic signs, an inability to rise and below average birthweight. The hind limbs and/or the forelimbs may be flexed or hyperextended and

neurologic examination may reveal ataxia, decreased patellar reflexes, and loss of conscious proprioception. Exophthalmia or an asymmetrical appearance in the eyes may be achieved and occasionally birth defects including hydrocephalus and a narrowing of the spinal cord may occur (Barr et al., 1991b, 1993; Dubey et al., 1998a, 1990a; Dubey and De Lahunta, 1993; De Meerschman et al., 2005).

2. Cause–effect relationship between *N. caninum* and abortion

Review of all published data indicates that *N. caninum* is a primary abortifacient in cattle (Dubey, 2003; Dubey et al., 2006). The lesions associated with *N. caninum* in the brains and the hearts of aborted fetuses can be severe enough to kill the fetus. In addition, there is recent evidence that an infection with *N. caninum* also triggers the release of pro-inflammatory cytokines and a Th1-type immuneresponse at the materno–fetal interface—a type of immuneresponse which could be detrimental for the pregnancy (Innes et al., 2005). Thus, cases may exist in which the fetus is not killed directly because of the *N. caninum*-associated lesions but by the shift from a beneficial Th2-type towards a detrimental Th1-type immunoresponse during gestation.

The seroprevalence of *N. caninum* in dairy cattle can be very high, approaching 100% in certain herds and the parasite is very efficiently transmitted from the infected dam to the fetus. These facts make it difficult to establish *N. caninum* as an abortifacient because an infection with the parasite could also be demonstrated in the aborted fetus or its mother even when *N. caninum* was not the cause of abortion.

To establish a cause–effect relationship it is important to use a comprehensive diagnostic approach utilizing serologic, immunohistochemical, and other methods to demonstrate the infection in the dam and the aborted fetus (Fig. 1). To achieve this objective, it is important to demonstrate *N. caninum* tachyzoites in lesions and exclude other causes of abortion (Anderson et al., 1991; Barr et al., 1991a; Wouda et al., 1997b). If the examination of maternal sera, fetal body fluids or fetal tissues is *N. caninum* positive by serology or PCR, the abortion might be associated with *N. caninum*, but it is important to rule out other potential causes at this stage (Fig. 1; diagnosis—step I). If lesions in the brains and hearts are very severe and *N. caninum* tachyzoites are demonstrable in lesions it might be justified at this stage to conclude that *N. caninum* is the very likely abortion cause (Fig. 1; diagnosis—step II). However, the decision whether lesions are or are not compatible with the life of a fetus is solely based on the experience of the examiner and there are to date no objective criteria. It has to be borne in mind that also in cases where no fatal lesions are observed *N. caninum* could have been the cause of abortion. Parasite loads in fetuses from epidemic abortion outbreaks may be higher than in those from endemic abortion and may be higher in fetuses aborted during the first and second trimester than in those that died in the last trimester (Collantes-Fernández et al., 2005; Wouda et al., 1997b). Thus, using less sensitive parasite detection methods under particular circumstances (endemic abortion, late gestation) may cause false negative results.

In cases when fetuses have not been submitted for examination or the fetal examination revealed no or only mild histologic lesions although there is evidence for an infection either by fetal or maternal serology or by a positive PCR it is important to examine the abortion problem within the group of animals with an abortion risk (Thurmond and Hietala, 1995). The rationale is to determine if among these dams with an abortion risk the *N. caninum* seroprevalence in aborting cows is higher than in the non-aborting (Fig. 1). The differences in seropositivity between aborting and non-aborting dams can be assessed by statistical procedures such as the chi-square or Fisher exact test. If there is a statistically significant association between a positive *N. caninum* antibody response and abortion it is justified to conclude that it is very likely that *N. caninum* was the cause of abortion (Fig. 1; diagnosis—step II). 'Dams at risk' have to be defined different in herds with endemic abortion problems versus herds with outbreaks. In endemic cases, the 'dams at risk' are defined as those which were pregnant during the period of time when the abortion problem occurred, e.g. during the past months or eventually years. In epidemic outbreaks 'dams at risk' might be defined as those which were pregnant for 58 to 260 days when the abortion storm started (Schares et al., 2002). It has to be borne in mind that in particular serological tests, aborting dams from herds with endemic bovine abortion have higher antibody

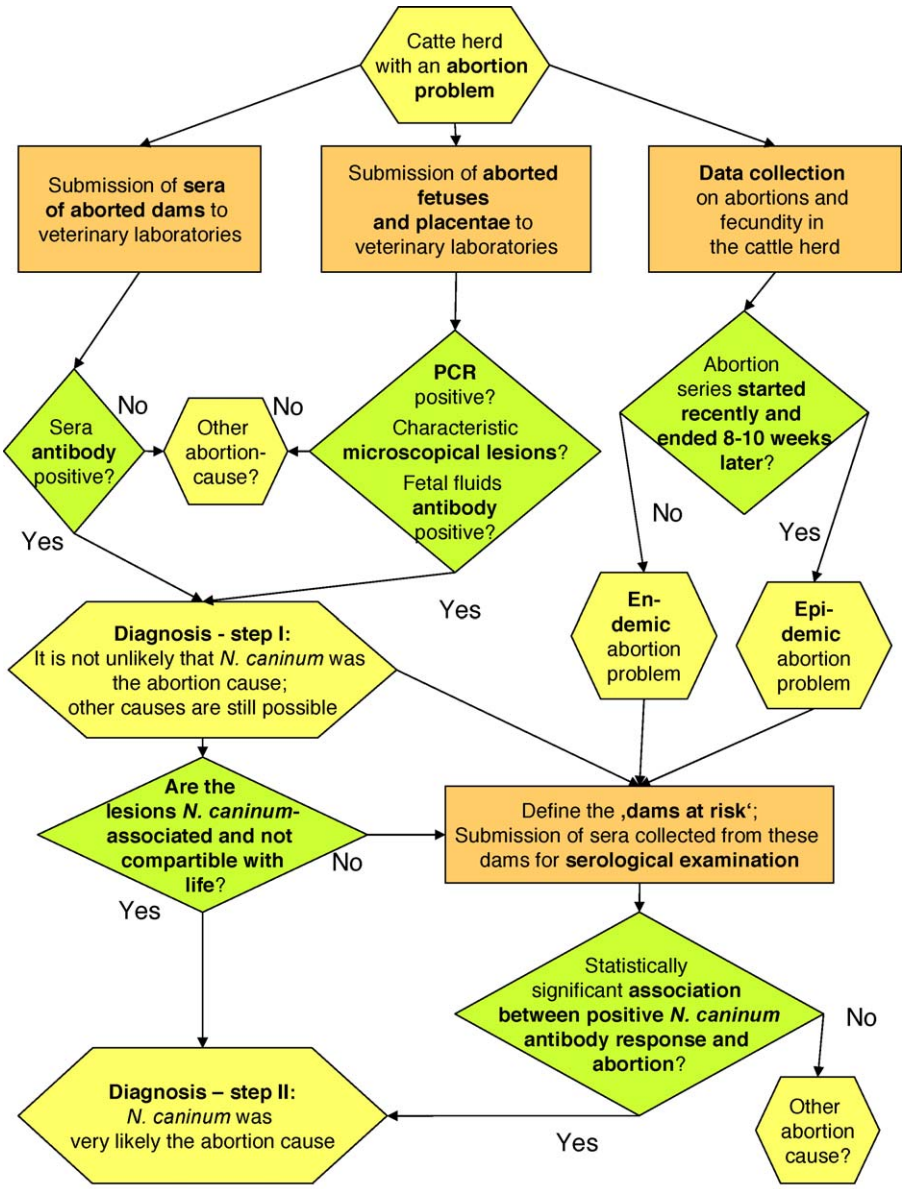

Fig. 1. Diagnosis of *N. caninum*-associated abortion. The serologic examination of maternal sera and fetal body fluids as well as the histological and the PCR analysis may provide first but not yet definitive evidence for a *N. caninum*-associated abortion (diagnosis—step I). If the lesions in fetal tissues are judged by a pathologist to be not compatible with life and if these lesions are immunohistochemically linked to *N. caninum* it may be justified to conclude that the abortion is caused by *N. caninum* (diagnosis—step II). The involvement of *N. caninum* in bovine abortion may also be confirmed by the observation of a statistically significant association between seropositivity and abortion within the group of dams with an abortion risk (‘dams at risk’). Definitions for ‘dams at risk’ are provided in the text.

levels than dams from herds afflicted by a recent abortion outbreak (Schares et al., 1999c; Schares et al., 2000)—possibly because dams in epidemic situations experienced primary infections very recently, i.e., their antibody response is still not completely developed. Using a less sensitive serological test in an epidemic situation may cause false negative serological results. In case of inconclusive serological results a

re-examination of aborting dams a few weeks later seems to be advisable (Schares and Rauser, unpublished).

3. Diagnosis of bovine neosporosis

3.1. Histopathologic

3.1.1. Lesions

Pathology is an important diagnostic procedure with lesions typically found in several tissues were reviewed in detail by Dubey et al. (2006). Salient features are repeated here.

3.1.1.1. Fetuses and stillborn calves. Degenerative to inflammatory lesions may be found throughout fetal tissues, but are most common in the central nervous system (CNS), heart, and the liver (Anderson et al., 1991; Barr et al., 1990, 1991a; Boger and Hattel, 2003; Dubey et al., 1998a, 1990b; Dubey et al., 2006; Hattel et al., 1998; Helman et al., 1998; Nietfeld et al., 1992; Wouda et al., 1997b). Gross lesions are rare, but may be present in heart, skeletal muscle, and the brain. Pale white foci may be present in skeletal muscles and the heart. Minute pale to dark foci of necrosis in the brain and hydrocephalus may occur (Dubey et al., 1998a). Often the fetuses are autolyzed and mummified. Focal areas of discoloration may be present in placental cotyledons (Fioretti et al., 2003).

Microscopic lesions may be present in many organs but are more common in the CNS, heart, liver, and examples are shown in Fig. 2.

Neural lesions are present in both the spinal cord and the brain and consist of non-suppurative encephalomyelitis characterized by multifocal non-suppurative infiltration, with or without multifocal necrosis and multifocal to diffuse non-suppurative leukocytic infiltration of the meninges. The characteristic lesion of neosporosis in the CNS consists of a focus of mononuclear cell infiltration around a central area of necrosis (Fig. 2A). Glial proliferation is more common in fetuses aborted in the third trimester (Dubey et al., 2006). Occasionally, there is calcification (Boulton et al., 1995).

Myocardial lesions are severe, but are often masked by autolysis. Typically, myocardial lesions consist of focal infiltration of mononuclear cells with minimal necrosis (Fig. 2C). Hepatic lesions consist of periportal infiltrations of mononuclear cells and variable foci of hepatocellular necrosis (Fig. 2D) with associated intrasinusoidal fibrin thrombi (Barr et al., 1990; Wouda et al., 1997b). Periportal hepatitis was more severe in epidemic versus endemic abortions, i.e. in exogenous versus endogenous transplacental infection. Collantes-Fernández et al. (2005) compared parasite load and severity of lesions in fetuses from epidemic and endemic abortions and fetuses at different stages of pregnancy. They concluded that the lesions and parasite loads in the heart, brain, lung, liver, kidney and lung were higher in fetuses from epidemic than endemic abortions. The stage of the pregnancy at the time of abortion also influenced the lesions and parasite loads; lesions and parasite load were higher in fetuses during the first and second trimester than the last trimester (Collantes-Fernández et al., 2005).

Placental lesions typically are confined to the cotyledons and consist of a focal area of necrosis and non-suppurative inflammation; tachyzoites are present in the trophoblasts (Barr et al., 1990, 1991a; Bergeron et al., 2001; Otter et al., 1995; Shivaprasad et al., 1989). The genesis of placental lesions in experimental infections was recently summarized (Dubey et al., 2006).

3.1.1.2. Neonatal calves. It seems that very few congenitally infected calves have clinical signs and it is difficult to find *N. caninum* histologically even in calves with clinical disease. In one study, *N. caninum* was found in histological sections of only one of the six calves that were born with neurological signs and that had precolostral *N. caninum* antibodies (De Meerschman et al., 2005). Encephalomyelitis was the predominant lesion in calves born live but clinically affected, or that developed clinical illness soon after birth and were necropsied by 2 weeks of age (Anderson et al., 1997; Barr et al., 1991b, 1993; De Meerschman et al., 2005; Dubey and Lindsay, 1996; Dubey et al., 1989, 1992; Magnino et al., 1999; O'Toole and Jeffrey, 1987; Parish et al., 1987; Peters et al., 2001a).

3.1.1.3. Adult cattle. N. caninum has not yet been identified in stained histological sections of tissues of cattle older than 2 months. Therefore, specificity of *N. caninum*-associated lesions has not been verified. Sawada et al. (2000) reported gliosis, and perivascular cuffs in the CNS, focal myositis and myocarditis, and

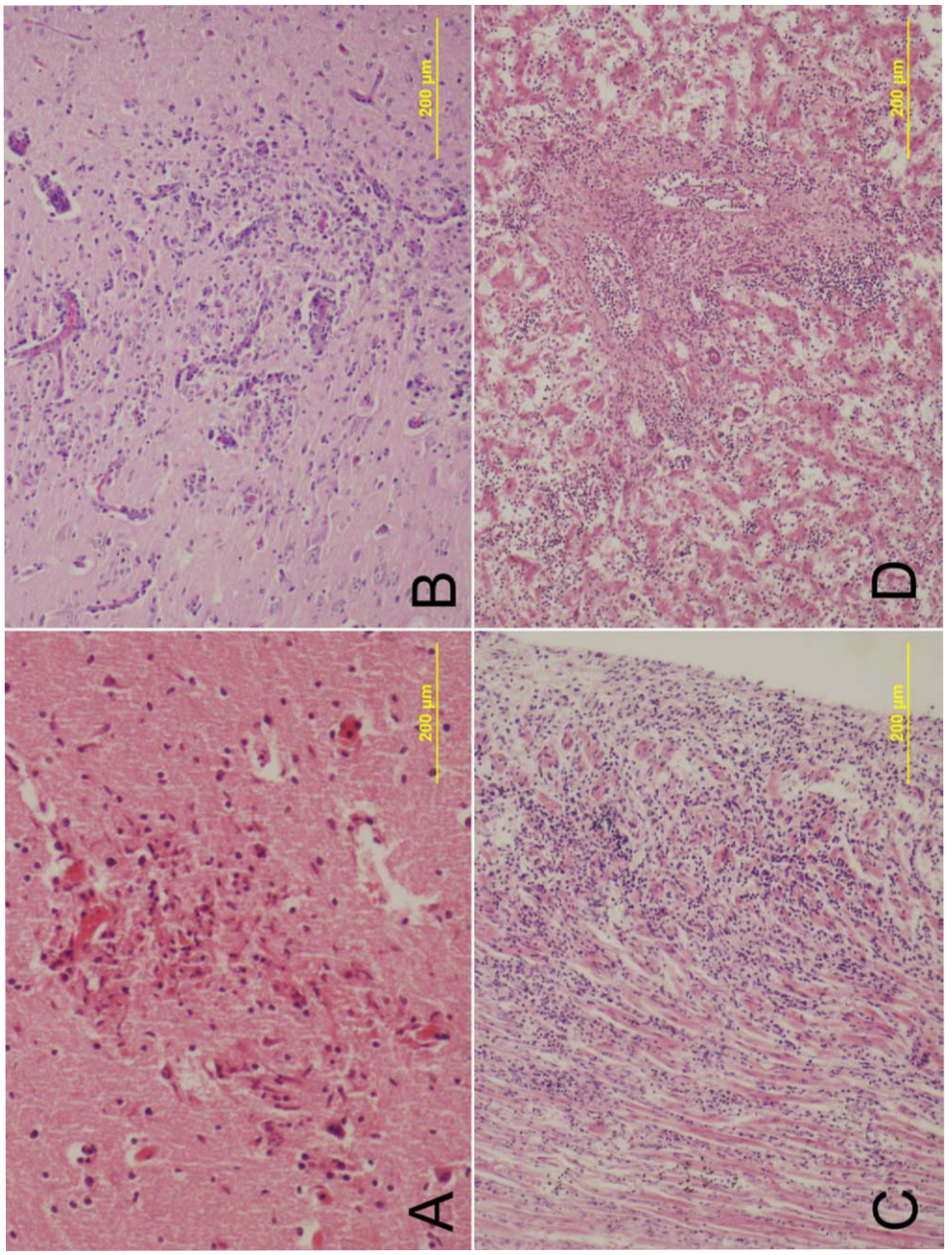

Fig. 2. Lesions in bovine fetuses typically associated with *N. caninum* infection in tissues stained with H and E. (A) Focal necrosis and mononuclear cell infiltration in the brain of a fetus. (B) Gliosis, perivascular infiltration of mononuclear cells, and neovascularization in a 3-day old calf. (C) Diffuse epimyocarditis in the heart of an aborted fetus. (D) Hepatitis characterized by mononuclear cell infiltration of parenchyma, especially periportal areas.

Table 1
Neospora caninum isolates from cattle

Country	Strain	Source	Primary isolation			Reference
			Trypsin treatment	Cell culture	Mice, gerbils	
Australia	NC-Nowra	Calf 7-day old	0.05% trypsin, 30 min	Vero	KO	Miller et al. (2002)
Brazil	BCN/PR3	Fetus	0.05% trypsin, 30 min	Vero	SW, gerbils	Locatelli-Dittrich et al. (2004)
Brazil	BNC-PR1	Calf 3-month old	0.05% trypsin, 30 min	Vero	No	Locatelli-Dittrich et al. (2003)
Italy	NC-PVI	Calf 45-day old	0.25% trypsin, 45 min	Vero	No	Magnino et al. (1999, 2000)
Italy	NC-PGI	Calf 8-month old	None	Vero	SW[a]	Fioretti et al. (2000)
Japan	JPA-1	Calf 2-week old	0.25% trypsin, 45 min	CPAE	Nude	Yamane et al. (1997)
Japan	BT-3	Adult cow	None	No	Nude	Sawada et al. (2000)
Korea	KBA-1	Calf 1-day old	0.25% trypsin, 30 min	Vero	No	Kim et al. (1998a, 2000)
Korea	KBA-2	Fetus	0.25% trypsin, 30 min	Vero	No	Kim et al. (1998b, 2000)
Malaysia	Nc-MalB1	Calf 1-day old (died)	None	No	BALB/c	Cheah et al. (2004)
New Zealand	NcNZ 1	Cow	2% trypsin, 30 min	Vero	No	Okeoma et al. (2004b)
New Zealand	NcNZ 2	Calf 2-day old	2% trypsin, 30 min	Vero	No	Okeoma et al. (2004b)
New Zealand	NcNZ 3	Stillborn	2% trypsin, 30 min	Vero	No	Okeoma et al. (2004b)
Portugal	NC-Porto1	Fetus	2% trypsin, 60 min	No	SW[c]	Canada et al. (2002a)
Spain	NC-SP-1	Fetus	2% trypsin, 60 min	No	SW[c]	Canada et al. (2004b)
Sweden	NC-SweB1	Stillborn	0.25% trypsin, 30 min[d]	Vero	No	Stenlund et al. (1997)
UK	NC-LivB1	Stillborn	0.5% trypsin, 30 min	Vero	No	Davison et al. (1999b)
UK	NC-LivB2	Fetus	NI[b]	NI	NI	Trees and Williams (2000)
USA	BPA-1	Fetus	0.05% trypsin, 1 h	No	No	Conrad et al. (1993a)
USA	BPA-2	Fetus	0.05% trypsin, 1 h	No	No	Conrad et al. (1993a)
USA	BPA-3	Calf 2-day old	NI[b]	CPAE	No	Marsh et al. (1995)
USA	BPA-4	Calf 6-day old	NI	CPAE	No	Marsh et al. (1995)
USA	NC-Beef	Calf	NI	NI	NI	McAllister et al. (1998, 2000)
USA	NC-Illinois	Calf	NI	NI	NI	Gondim et al. (2002)

[a] Swiss Webster, 2.5 mg methylprednisolone acetate.
[b] NI = No information provided.
[c] Dexamethasone 10 μg/ml.
[d] Parasites concentrated by centrifugation in 30% Percoll.

infiltrates of mononuclear cells in the liver and kidney of a cow from whom they isolated viable *N. caninum* (Table 1). Fioretti et al. (2000) reported focal myocarditis but no encephalitis in the 8-month old calf from whom they had isolated *N. caninum* (Table 1). They also found tachyzoites in sections of the heart that reacted with *N. caninum* antibodies. However, a closer examination of the illustrations in the paper suggests that the parasites most likely observed are sarcocysts of *Sarcocystis*.

3.1.2. Demonstration of N. caninum in hematoxylin and eosin (H and E) sections

The number of *N. caninum* found in bovine tissues is typically low even in well preserved dead fetuses to the extent that it is difficult to recognize tachyzoites in routine H and E stained sections. The parasite-initiated response that kills the host cells also kills the parasite and therefore it is rare to find well preserved intact tachyzoites in dead fetuses (Dubey and Lindsay, 1996; Dubey et al., 2006). The genesis of lesions and the appearance of *N. caninum* tachyzoites and tissue cysts in H and E stained sections were illustrated recently (Dubey et al., 2006). In H and E sections the tachyzoites are often round to slightly elongate and it is important to look for the vesicular nucleus to distinguish them from degenerating host cells. Rarely the tachyzoites are cut longitudinally to be seen as crescentric (Dubey et al., 2006). In bovine tissues *N. caninum* tissue cysts are 5–50 μm in diameter and the thickness of the cyst wall varies from <1 to 2.5 μm; thickness of the cyst wall is independent of the size of the tissue cyst. Bradyzoites in tissue cysts have a terminally located nucleus and they stain red with periodic acid Schiff (PAS) reaction (Dubey et al., 2002).

If apicomlexan-like protozoa are found in the brain of bovine aborted fetuses, they can be assumed to be *N. caninum*. *Toxoplasma gondii* and *Sarcocystis cruzi*

are the two other related protozoans that are abortifacient. *S. cruzi* schizonts occur in endothelial cells and have immature stages consisting of multiple nuclei without merozoites whereas in *N. caninum* and *T. gondii* there is no immature stage; ultrastructurally *Sarcocystis* merozoites lack rhoptries. *T. gondii* is morphologically similar to *N. caninum*. Although *T. gondii* DNA was detected in (Ellis, 1998; Gottstein et al., 1998; Sager et al., 2001) and *T. gondii* could be isolated from aborted bovine fetuses (Canada et al., 2002b) there is no documented case of a proven *T. gondii*-associated abortion in cattle.

3.1.3. Immunohistochemical staining

Immunohistochemical staining is more reliable to demonstrate *N. caninum* in lesions than the conventional H and E staining and all tissues showing lesions should be treated immunohistochemically for *N. caninum* to exclude neosporosis (Boger and Hattel, 2003). Both polyclonal and monoclonal antibodies specific to *N. caninum* can be used (Cole et al., 1994; Lindsay and Dubey, 1989a) and both polyclonal and monoclonal *N. caninum* antibodies are commercially available. Polyclonal antibodies made in rabbits seem to be more reliable than the mouse-derived monoclonal antibodies for diagnostic purposes (Schares and Dubey, unpublished observation). Cross-reactivity of *N. caninum* antibodies to related apicomplexans, *T. gondii*, and *Sarcocystis* spp. is not a major issue because these protozoans are rarely associated with abortion in cattle (Anderson et al., 1991; Canada et al., 2002b). However, a recent interlaboratory comparison of immunohistochemical protocols revealed false-positive *N. caninum* reactions in tissue sections of an *T. gondii* infected animal (Van Maanen et al., 2004).

Groups of tachyzoites often cannot be distinguished from tissue cysts based on immunohistochemistry unless bradyzoite-specific antibodies are used (McAllister et al., 1996b). Tissues with high concentration of peroxidase, especially placenta, should be treated with trypsin or pepsin and the procedure used in our laboratory is outlined in Dubey et al. (2001). The diagnosis should not be made unless parasite outlines are visible because diffuse staining may be non-specific.

By immunohistochemical staining *N. caninum* is most often demonstrable in the brain and heart and rarely in other organs, including the placenta. In the study by Wouda et al. (1997b), *N. caninum* was found in the brain of 71 (89%), heart of 11 (14%) and in the liver of 21 (26%) of 80 fetuses. Wouda et al. (1997b) also found that there were more *N. caninum* organisms in epidemic versus endemic cases of abortion. Occasionally, *N. caninum* may be demonstrated only in extraneural tissues (Boger and Hattel, 2003).

3.2. Demonstration of viable N. caninum

Attempts at isolation of viable *N. caninum* by bioassay in mice or cell culture have been largely unsuccessful. *N. caninum* has been isolated from seven aborted fetuses, three stillborns, nine 1–14-day old clinical calves, one 45-day old clinical calf, one 3-month old blind calf, one 8-month old asymptomatic calf, and two adult asymptomatic cows (Table 1). In addition to the reports in Table 1, *N. caninum* was isolated in cell cultures inoculated with homogenates of brain and spinal cords of two neonatal calves with precolostral antibodies; no other details were provided (Anderson et al., 1997). Many attempts to isolate viable *N. caninum* have been unsuccessful because most parasite stages die within the fetus when it succumbs to the infection. Probably for this reason, Conrad et al. (1993a) recovered *N. caninum* from only two of 49 histologically confirmed *N. caninum*-infected fetuses, and in both instances tissue cysts were present. It is easier to isolate *N. caninum* from neural tissues of congenitally infected full-term calves, perhaps because tissue cysts are likely to be present and tissue cysts are relatively more resistant to autolysis than tachyzoites. For concentration of the parasites, host tissue (brain, spinal cord) can be digested in trypsin or pepsin. It should be borne in mind that tachyzoites can be killed by pepsin whereas bradyzoites may not be, depending on the duration of digestion process. As can be seen from the data in Table 1 there is no standardized method to isolate *N. caninum* from bovine tissues. We homogenize tissues in saline (0.85% NaCl) in a blender to prepare a 20% weight/volume suspension and add an equal volume of 0.5% trypsin in saline or acid pepsin (Dubey, 1998) and incubate at 37 °C for 30–60 min on a shaker. After centrifugations and washings the sediment is suspended in antibiotic saline (1000 units penicillin, 100 μg streptomycin/ml of saline). The pepsin procedure has the advantage that the acid kills many bacteria,

and repeated centrifugations are not needed because the acid can be neutralized with sodium bicarbonate as described (Dubey, 1998); this procedure is recommended for isolating *Neospora* from the tissues of chronically infected animals. The trypsin procedure is more labour intensive because repeated washings are necessary to remove the trypsin.

There are no data on the quantitative survival of *N. caninum* tachyzoites and bradyzoites in different concentrations of trypsin or pepsin. Although *T. gondii* tachyzoites and bradyzoites can survive in acid pepsin and 1% trypsin for more than 2 h there is 1–2 log loss of infectivity after 1–2 h of incubation (Sharma and Dubey, 1981). Therefore, we recommend the incubation of host tissue not longer than 60 min in final concentration of trypsin not higher than 0.25%.

Neospora parasites can be separated from neural tissue by Percoll gradient, a technique first developed by Cornelissen et al. (1981) to separate *T. gondii* tissue cysts from mouse brain and later applied to separate *N. caninum* from the host tissue (Fioretti et al., 2000; McGuire et al., 1997; Stenlund et al., 1997). Tissue cysts microisolated from brain homogenate can be inoculated individually into gamma interferon gene knockout (GKO) mice to obtain cloned parasites (Dubey et al., 2004). However, the infectivity of *N. caninum* bradyzoites to cell cultures or mice may be lower than that of tachyzoites. Additionally, bradyzoites should be released from tissue cysts by pepsin or trypsin before inoculation into cell cultures because intact tissue cysts are not infective to cell cultures (J.P. Dubey, unpublished observation).

Numerous mammalian cell lines have been used to grow *N. caninum* in cell cultures (Lindsay and Dubey, 1989c) and the procedures used to isolate viruses in diagnostic laboratories are suitable to grow *N. caninum*. Although most authors used VERO cells to grow *N. caninum* from bovine tissues (Table 1), *N. caninum* has no recognized cell culture preference (Lei et al., 2005). Usually, the cell cultures are incubated with the tissue homogenate for 1 h and then replaced with cell culture medium. It is important to observe the cell culture flask for 2 months because most *N. caninum* strains are slow growing and tachyzoites may not be visible microscopically for 60 days from the time of seeding with the homogenate.

Immunosuppressed mice are more efficient than the cell culture for obtaining viable *N. caninum*. Inbred mice (e.g. BALB/c) are more susceptible to *N. caninum* infection than outbred mice (Dubey et al., 1988; Lindsay et al., 1995; Long and Baszler, 1996; Long et al., 1998). The GKO mice are more suitable for *N. caninum* infection than the nude mice (Dubey et al., 1998b); however the susceptibility of different lines of GKO mice has not been compared. We have used the BALB/c derived GKO mice (C.129S7(B6)-*Ifng*^{tm1Ts}/J; The Jackson Laboratory). Nude mice and SCID mice are also susceptible to *N. caninum* (Yamane et al., 1998). *N. caninum* has also been cultivated in cortisone-treated outbred mice (Lindsay and Dubey, 1989b). Mice can be immunosuppressed with methylprednisolone acetate or cortisone acetate by injection or with dexamethasone (Sigma) orally in drinking water (1 μg/ml) from the day of inoculation with tissue homogenate until the mice get ill. In immunosuppressed mice that die 2–4 weeks post inoculation (p.i.), *Neospora* is usually found in the liver, lung, and the heart. Five weeks p.i. *Neospora* are usually in the brain. GKO mice inoculated with certain strains of *N. caninum* may not get ill until 8 weeks p.i. (Vianna et al., 2005). GKO mice infected with *N. caninum* develop antibodies that can be detected by various serological tests (Schares et al., 2005b) and thus it is advantageous to use GKO mice instead of Nude mice; the latter may not develop antibodies. *N. caninum* has also been isolated from bovine tissues in mouse sarcoma cells grown in the peritoneal cavity of immunosuppressed mice; this method is labour intensive but very efficient (Romand et al., 1998; Canada et al., 2002a, 2002b).

The Mongolian gerbil (*Meriones unguiculatus*) is also susceptible to *N. caninum* infection but they are not reliable because *N. caninum* can spontaneously disappear in gerbils (Cuddon et al., 1992; Dubey and Lindsay, 1996; Dubey and Lindsay, 2000; Dubey et al., 2004; Gondim et al., 1999; Ramamoorthy et al., 2005; Schares et al., 2005b). Pipano et al. (2002) quantitatively compared the susceptibility of *Meriones tristrami* and the sand rat (*Psammomys obesus*) to *N. caninum* tachyzoites. Both of these rodent species inoculated with as few as 10 *N. caninum* tachyzoites became infected and developed clinical signs.

The isolation of *N. caninum* from an 8-month old calf by Fioretti et al. (2000) is important for several reasons. First, the dam was diagnosed as having recently acquired *N. caninum* infection based on the

detection of high IgG and IgM antibodies at 230 days of gestation. Second, although a healthy calf was born at 280 days of gestation, the placenta contained inflammatory foci and viable *N. caninum* was isolated by bioassay in mice. Third, thick-walled *N. caninum* tissue cysts were found directly in the brain of the calf after it was killed at 8 months of age and viable *N. caninum* was recovered in mice and in cell culture, even though the calf was clinically normal.

The isolation of *N. caninum* from the brain of a 2-year old cow by Sawada et al. (2000) was the first isolation of *N. caninum* from an adult cow. The cow had twice aborted *N. caninum*-infected fetuses, and was killed 24 days after the second abortion. Although protozoa were not demonstrable in histologic sections of the infected brain, it had a mild non-suppurative encephalitis; the cow was otherwise clinically normal.

The second isolate of *N. caninum* from adult cattle was from a 2-year old Friesian cow from New Zealand (Okeoma et al., 2004b). This cow when sampled from the day 150 of pregnancy onwards had persistent *N. caninum* antibodies throughout the gestation. The cow and her 2-day old calf were killed and *N. caninum* was isolated from both animals. The calf had precolostral *N. caninum* antibodies and both the dam and the calf were apparently asymptomatic.

3.3. Serologic diagnosis

Serological tests have the advantage that they can be applied antemortem and may provide information on the stage of infection. In calves or adult cattle, a few days after primary infection specific IgM and IgG antibodies appear. While the specific IgM levels peak after 2 weeks of infection and declining below the detection limits in a *Neospora* agglutination test (NAT) at 4 weeks of infection again (De Marez et al., 1999), IgG levels increase during the first weeks up to 3–6 months after experimental primary infection (Conrad et al., 1993b; De Marez et al., 1999; Dubey et al., 1996; Schares et al., 1999c, 2000; Trees et al., 2002; Uggla et al., 1998; Williams et al., 2000). After tachyzoite or oocyst induced primary infection, an initial rise in specific IgG_1 is followed by a slightly delayed surge of IgG_2 (Andrianarivo et al., 2001; De Marez et al., 1999; Williams et al., 2000). No elevated IgA levels were observed in calves experimentally infected by oocysts (De Marez et al., 1999). Levels of specific antibodies may persist for the life but fluctuate and sometimes are below the detection limits of serological tests (for more details see below).

After primary *N. caninum* infection the avidity of specific antibodies increases over time (Björkman et al., 1999, 2005) and this can provide to some extend information on the duration of a primary infection. Several avidity assays have been developed to differentiate low avidity IgG responses (indicative for a recent primary infection, approximately of 2-month duration) from high avidity IgG responses (indicative for a chronic infection). Usually high avidity IgG responses are observed in cattle naturally infected for more than 6 months (Björkman et al., 1999, 2003). In field studies low avidity IgG responses could be linked to *N. caninum*-associated abortion epidemics suggesting that a recent primary infection was the cause of abortion (Jenkins et al., 2000; McAllister et al., 2000; Sager et al., 2005; Schares et al., 2002).

After vaccination of cattle with inactivated *N. caninum* vaccines the interpretation of serologic data may become difficult. A vaccine consisting of killed cell-culture-derived tachyzoites and adjuvant is commercially available and is used in several countries (e.g. in the USA (Estill, 2004), in Costa Rica (Romero et al., 2004) and in New Zealand (Heuer et al., 2003)). Another non-commercial vaccine also consisting of cell-culture-derived tachyzoites and adjuvant was recently used for experimental vaccination of cattle in Agentina (Moore et al., 2005). Animals inoculated with these vaccines developed antibody responses which were similar to those of cattle naturally infected with *N. caninum* (Andrianarivo et al., 2001; Choromanski and Block, 2000; Moore et al., 2005). Therefore it was recently proposed to develop marker vaccines together with companion serological tests to distinguish the antibody responses in vaccinated from those in naturally exposed cattle (Conraths and Ortega, 2005).

3.3.1. Serological assays

Several assays are available for detecting antibodies to *N. caninum* in cattle (Table 2). All of these assays are based on tachyzoite antigens. Assays based on antigens of bradyzoites or sporozoites are not yet available.

Some serological tests utilize air dried or fixed *N. caninum* tachyzoites in the indirect fluorescence antibody test (IFAT) or the NAT (Table 2). Both

Table 2
Serological assays developed to detect antibodies in *Neospora caninum*-infected cattle

Type of test	Test characteristics	Reference	Comment
Direct agglutination test	Whole fixed tachyzoite	Packham et al. (1998)	Cell-culture-derived
		Romand et al. (1998)	Mouse-derived
IFAT	Whole fixed tachyzoites	Conrad et al. (1993b)	Air-dried
		Buxton et al. (1997)	Formaldehyde fixed, air-dried
		Schares et al. (1998)	Air-dried, acetone fixed
ELISA	Whole tachyzoite lysate antigen indirect ELISA (various lysate protocols)	Paré et al. (1995)	Extracted with PBS, kinetic ELISA
		Dubey et al. (1996)	Extracted with PBS
		Reichel and Drake (1996)	Commercial antigen
		Osawa et al. (1998)	Extracted with distilled water
		Gottstein et al. (1998)	Extracted with PBS
		Wouda et al. (1998b)	Extracted with PBS, 1% Triton X-100
		Bae et al. (2000)	
	Fixed whole tachyzoite indirect ELISA	Williams et al. (1997)	Formaldehyde fixed
	ISCOM antigen indirect ELISA	Björkman et al. (1997)	Based on Björkman et al. (1994)
	Recombinant antigen indirect ELISA	Lally et al. (1996b)	rNcGRA6 and rNcGRA7
		Louie et al. (1997)	rNcGRA7 and recombinant subtilisin-like serin protease
		Nishikawa et al. (2001)	rNcSRS2
		Howe et al. (2002)	Truncated rNcSRS1
		Chahan et al. (2003)	Truncated rNcSRS1
		Ahn et al. (2003)	Truncated rNcSRS2
		Jenkins et al. (2005)	rNcGRA6; HPLC purified
		Gaturaga et al. (2005)	Truncated rNcSRS2
	Single native antigen indirect ELISA	Schares et al. (2000)	NcSRS2
	Antigen-capture indirect ELISA	Schares et al. (1999c)	Polyclonal antiserum for antigen capture
	Antigen-capture competitive inhibition ELISA	Dubey et al. (1997)	Monoclonal antibody for antigen capture
		Baszler et al. (2001)	
	Competitive inhibition ELISA	Baszler et al. (1996)	Based on a monoclonal antibody
		McGarry et al. (2000)	Based on a polyclonal antibody
	Avidity ELISA	Björkman et al. (1999)	ISCOM incorporated antigen
		Maley et al. (2001)	Whole tachyzoite lysate
		Schares et al. (2002)	NcSRS2
		Sager et al. (2003)	Whole tachyzoite lysate
	Bulk-milk ELISA	Björkman et al. (1997)	ISCOM incorporated antigen
		Schares et al. (2003)	NcSRS2
		Bartels et al. (2005)	Whole tachyzoite lysate
Immunoblot	Reduced whole tachyzoite antigen	Bjerkås et al. (1994)	
	Non-reduced whole tachyzoite antigen	Bjerkås et al. (1994)	
	Avidity Western blot	Aguado-Martinez et al. (2005)	
RIT (rapid immunochromatographic test)	Recombinant antigen RIT	Liao et al. (2005)	Truncated rNcSRS1

methods detect antibodies to the tachyzoite surface which obviously provides many antigens which are *Neospora* specific as demonstrated by the species specificity of monoclonal antibodies developed against the outer membrane antigens of tachyzoites (Baszler et al., 1996; Björkman and Hemphill, 1998; Howe et al., 1998; Schares et al., 1999b). It is important to note that the detection of antibodies by IFAT and also by other assays can give false-positive results if fetal bovine serum (FBS) in the cell culture medium often used to grow *N. caninum* contains antibodies to *N. caninum*; most batches of commercial FBS in the US and in Europe have antibodies to *N. caninum*.

The first studies to describe the use of either reduced or non-reduced antigens in immunoblots (IBs) to diagnose *N. caninum* infections were by Barta and Dubey (1992) and Bjerkås et al. (1994). Different immunodominant *N. caninum*-specific antigens were identified, among them a 19, 29, 30, 33, and a 37 kDa antigen (Fig. 3). Stronger reactions are observed against non-reduced antigens, suggesting that conformational epitopes are predominantly involved in the *N. caninum*-specific antibody response (Fig. 3). Working groups using non-reduced antigens (Atkinson et al., 2000; Paré et al., 1995; Stenlund et al., 1997) report fewer on cross-reactivities between sera from animals infected with *N. caninum*, *T. gondii* or *Sarcocystis* sp. than researchers who used reduced antigens (Baszler et al., 1996; Harkins et al., 1998). A potential reason for this is that in *N. caninum* conformational epitopes might be more species specific than linear epitopes.

Several enzyme-linked immunosorbent assays (ELISAs) have been described to examine bovine sera for *N. caninum* antibodies (Table 2). These ELISAs utilize either whole or fixed *N. caninum* tachyzoites, aqueous or detergent-soluble total tachyzoite extracts, single native antigens or recombinant tachyzoite antigens. Different methods have been used to solubilize tachyzoite antigens. Recently some of these methods were comparatively evaluated (Zintl et al., 2006). Based on monoclonal and polyclonal antibodies, competitive inhibition ELISAs (CI-ELISAs) have been developed which detect antibodies to *N. caninum*-specific epitopes.

Most of the commercialized ELISAs to detect *N. caninum*-specific antibodies are based on total

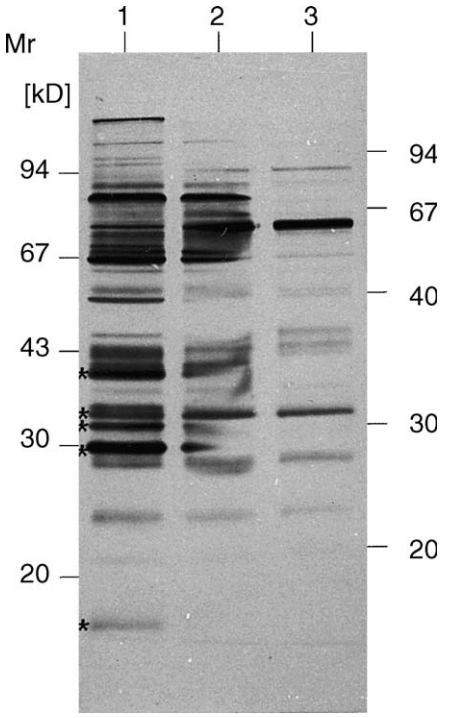

Fig. 3. Reactivity of a *N. caninum* positive bovine serum against *N. caninum* tachyzoite antigens separated under non-reducing (lane 1) and reducing conditions (lane 3). Note that non-reduced antigens were loaded also in lane 2. However, during the gel run the agent used for the reduction of disulfide bonds (2-mercaptoethanol) moved from lane 3 into lane 2 and led to a partial reduction of the antigens. Since exactly the same antigen concentration had been loaded to each of the lanes, the result clearly demonstrates that the reactivity against immunodominant antigens (marked with * is strongly reduced when the antigens are denatured by disulfide bond reducing agents).

tachyzoite lysate antigen (Table 3). However, there are also commercialized tests using fixed *N. caninum* tachyzoites (Williams et al., 1999), ISCOM incorporated tachyzoite antigens or native affinity-purified NcSRS2 (Von Blumröder et al., 2004).

A number of recombinant antigens of potential diagnostic value are published (Table 2). In the future, recombinant antigens might become more important, because they can be produced easier in large quantities and better standardized for the production of serological assays (e.g., for rapid immunochromatographic tests (RITs) which need more antigen than ELISAs). Recently, Liao et al. (2005) reported on a non-commercial RIT using a recombinant surface antigen of *N. caninum* tachyzoites (NcSRS1) as an

Table 3
Commercially available serological diagnostic tests also used for research purposes

Test	Format	Antigen preparation	Company	References containing validation data
BIOVET *Neospora caninum*	Indirect ELISA	Sonicate lysate of tachyzoites	BIOVET Laboratories, Canada	Wu et al. (2002), Waldner et al. (2004)
CHEKIT *Neospora* Dr. Bommeli/IDEXX	Indirect ELISA	Detergent lysate of tachyzoites	IDEXX Laboratories, The Netherlands	Von Blumröder et al. (2004)
CIVTEST BOVIS NEOSPORA	Indirect ELISA	Sonicate lysate of tachyzoites	HIPRA, Spain	Von Blumröder et al. (2004)
Cypress Diagnostics C.V. *Neospora caninum*	Indirect ELISA	Detergent lysate of tachyzoites	Cypress Diagnostics, Belgium	Von Blumröder et al. (2004)
HerdChek IDEXX	Indirect ELISA	Sonicate lysate of tachyzoites	IDEXX Laboratories, USA	Bartels et al. (2005), Paré et al. (1995)[a], Reichel and Pfeiffer (2002), Schares et al. (1999a), Schares et al. (2004b), Von Blumröder et al. (2004), Wouda et al. (1998b), Wu et al. (2002)
MASTAZYME *Neospora*	Indirect ELISA	Whole tachyzoites	MAST GROUP, United Kingdom	Williams et al. (1997), Schares et al. (1999a), Wouda et al. (1998b), Von Blumröder et al. (2004)
Neospora caninum blocking ELISA	Competitive ELISA	No information	Institut Pourquier, France	Hall et al. (2005)[b]
P38-ELISA	Indirect ELISA	Affinity-purified native surface antigen of tachyzoites (NcSRS2)	AFOSA GmbH, Germany	Schares et al. (2000)[a], Von Blumröder et al. (2004)
ImmunoComb bovine *Neospora* antibody	DOT-ELISA	No information	Biogal, Israel	Toolan (2003)[b]
SVANOVIR *Neospora*-Ab ELISA	Indirect ELISA	ISCOM incorporated antigen	SVANOVA Biotech AB, Sweden	Björkman et al. (1997)[a], Frössling et al. (2003)[a], Varcasia et al. (2006), Hůrková et al. (2005)
VMRD *Neospora caninum* cELISA	Competitive ELISA	GP65 surface antigen of tachyzoites	VMRD, USA	Baszler et al. (2001), Jakubek and Uggla (2005)
VMRD *Neospora caninum* FA substrate slide[c]	IFAT	Whole tachyzoites	VMRD, USA	Frössling et al. (2003), Reichel and Drake (1996), Reichel and Pfeiffer (2002), Schares et al. (1999a)

[a] This reference provides no validation data on the commercial test but on the in-house test the commercial product is based on.
[b] Unpublished validation results are mentioned in this reference.
[c] Only IFAT slides are supplied.

antigen. To our knowledge, as yet, no commercialized ELISAs are available based on recombinant antigens. Recently, it was shown that insufficient purification of a recombinant dense granule antigen (rNcGRA7) for ELISA diagnosis caused false-positive reactions (Jenkins et al., 2005).

A number of protocols were developed to use the above mentioned serum ELISAs also for the examination of individual bovine milk and bovine bulk-milk samples (Björkman et al., 1997; Chanlun et al., 2002; Frössling et al., 2006; Schares et al., 2003, 2005a) including some of the commercialized ELISAs (Bartels et al., 2005; Hůrková et al., 2005; Schares et al., 2004b; Varcasia et al., 2006) (Tables 2 and 3). Protocols were also developed to use some of the ELISAs mentioned above in epidemiological studies to examine the avidity of a *N. caninum*-specific antibody response (Table 2).

We are aware on a number commercially available *N. caninum* antibody tests. Validation data on these tests are only partially published (Table 3). The most of these tests are ELISAs. However, also IFAT slides are commercially available. A commercialized DOT-ELISA might be applicable under field conditions.

3.3.2. Selection of serological tests and cut-offs

Each of the different serological methods listed above can be applied for different purposes. However, it has to be stressed that it is not advisable to use serological tests before evaluating them for the application in which they will be used (Greiner and Gardner, 2000). Selecting an appropriate cut-off (Álvarez-García et al., 2003; Jenkins et al., 2002) is critical for any serological assay used for bovine neosporosis. However, for some application it might be advisable, not only to select an appropriate cut-off, but also to change the test protocol (e.g. the type of antigen, antigen concentration, serum and conjugate dilution, specificity of conjugate for a particular isotype). Most of the tests described above were developed to diagnose bovine abortion. However, an other important application is for detecting infected cattle (e.g. calves, replacement heifers, bulls) to identify and later remove *N. caninum*-infected animals from herds (Hall et al., 2005), to prevent the new introduction of infected animals into herds, or to exclude infected dams from embryo-transfer (Baillargeon et al., 2001; Landmann et al., 2002). Serological tests to identify infected cattle may require a higher sensitivity i.e., lower cut-offs than those meant to diagnose bovine abortion (Álvarez-García et al., 2003; Schares et al., 1999a).

One of the major problems to define appropriate cut-offs to identify infected cattle is that there is no appropriate gold standard to define a true-positive or true-negative reference group. Different approaches are followed to overcome this problem and to validate serological tests for the purpose of detecting *N. caninum* infection or to diagnose bovine neosporosis (Álvarez-García et al., 2003; Baszler et al., 2001; Canada et al., 2004a; Frössling et al., 2003; Venturini et al., 1999; Von Blumröder et al., 2004; Williams et al., 1999). For future validations gold-standard-free approaches may become more and more important (Frössling et al., 2003).

In the past there was some debate on appropriate cut-off titres for IFATs. However, a specific situation exists regarding the selection of appropriate cut-offs for this assay. Because the IFAT titers are largely dependent on the quality of the equipment used for fluorescence microscopy, it is often impossible to standardize the IFAT test results among different laboratories. Consequently, a cut-off titer appropriate in one particular laboratory might not be suitable in another. Consequently, each laboratory should establish an own IFAT cut-off and not rely on those reported in literature.

3.3.3. Serological diagnosis of infection and neosporosis-associated mortality

3.3.3.1. Examination of individual breeding dams. After the occurrence of bovine abortion, stillbirth or neonatal mortality on a farm, a serological examination of the afflicted dams may provide information if these dams are infected with *N. caninum* and, if so, whether they experienced a recent or have had a chronic infection (see below). Most cows that have a *N. caninum*-infected fetus are seropositive at the time of abortion (De Meerschman et al., 2002; Otter et al., 1997; Söndgen et al., 2001; Wouda et al., 1998a) or after calving (Anderson et al., 1997; Davison et al., 1999c; Ho et al., 1997a). Consequently, a negative serological test result for the dam makes it unlikely that *N. caninum* was involved in abortion, stillbirth or neonatal mortality. In some cases, however, *N. caninum*-infected fetuses were aborted or positive

calves born by seronegative dams (Davison et al., 1999b, 1999c; Sager et al., 2001). This might be due to fluctuating antibody levels (Conrad et al., 1993b). These are observed during pregnancy (Dannatt, 1998; Fioretti et al., 2003; Guy et al., 2001; Paré et al., 1997; Quintanilla-Gozalo et al., 2000; Stenlund et al., 1999), around abortion (Guy et al., 2001; Schares et al., 1999c; Wouda et al., 1998b) or after calving (Stenlund et al., 1999). The antibody levels may drop below the detection limit of less sensitive tests and cause false negative results (Dannatt, 1998; Dijkstra et al., 2003; Wouda et al., 1998b). Although infected, some animals do not develop antibodies at all, or the antibodies are not detectable by particular tests as it was reported in a calf experimentally infected by oocysts (De Marez et al., 1999). The presence of antibodies to *N. caninum* in the serum of a dam allows no definitive diagnosis because only a low proportion of infected dams abort, and most of their calves are born infected but healthy (Guy et al., 2001; Paré et al., 1997; Thurmond and Hietala, 1997; Wouda et al., 1998a). Therefore, a dam may have antibodies against *N. caninum*, although abortion, stillbirth or birth of a weak calf may have had another cause. Positive serological testing of individual dams only allows one to suspect *N. caninum* infection but is no proof that *N. caninum* was involved in the reproductive failure.

3.3.3.2. Serological examinations on a herd level. A more definitive diagnosis can be achieved when the problem (abortion, stillbirth, neonatal mortality) is examined including all dams at risk or is examined on a herd level. To clarify e.g. the reason for an abortion problem in a herd, a seroepidemiological approach has been proposed by Thurmond and Hietala (1995). The rationale is to determine by statistical methods if the proportion of seropositivity in aborting cows is higher than that in non-aborting cows (i.e. to determine whether abortion is statistically associated with seropositivity to *N. caninum*). It must be stressed that only serological results that have been obtained from dams at risk (i.e. those dams that were pregnant during the period of time when the abortion problem occurred) should be included in the analysis (Section 2). In endemic cases, the period during which pregnant dams have an abortion risk may last several months up to years (Davison et al., 1999a; Schares et al., 1998, 2002; Thurmond et al., 1997), while in epidemic cases it may last only a few weeks (e.g. McAllister et al., 1996a; Thornton et al., 1994; Wouda et al., 1999; Yaeger et al., 1994).

Animals that abort due to neosporosis often have higher *N. caninum*-specific antibody levels than infected but non-aborting dams (Dubey et al., 1997; McAllister et al., 1996a; Quintanilla-Gozalo et al., 2000; Schares et al., 1999c, 2000; Waldner et al., 1998). The same is true for cows that transplacentally transmit the infection to their calves (Guy et al., 2001). Those serological tests that have a cut-off not adjusted to detect all dams that are infected with *N. caninum* but adjusted to detect those animals with elevated antibody levels are useful to demonstrate association between seropositivity and abortion (Schares et al., 1999a) or seropositivity and vertical transmission (Davison et al., 1999c). In particular ELISAs, aborting dams from herds with endemic bovine abortion appeared to have higher antibody levels than dams from herds afflicted by recent epidemic abortion (Schares et al., 1999c, 2000). This effect was also observed with a number of commercialized tests (Schares, unpublished) and has to be taken into account when selecting an appropriate cut-off to diagnose bovine abortion. Serological tests have to be evaluated with positive abortion sera from both, epidemic and endemic situations.

It may become necessary to make supplementary analyses on a herd level to identify the predominant route by which the cattle became infected with *N. caninum*. For this purpose avidity ELISAs could be used (Table 2). As mentioned above, these tests measure the avidity of *N. caninum*-specific IgG and are able to find indications for recent infection on a herd level. However, the interpretation of avidity test results for individual animals should be done with care, because individual animals can maintain a low avidity antibody response although infected for several years (Björkman et al., 2003). In addition, the analysis of dam–daughter pairs may provide information whether the infection is predominantly transmitted vertically in a herd (Thurmond et al., 1997). In herds in which there is a positive association regarding the seropositivity of dams and their daughters the predominant route of infection seems to be vertical transmission. Further information on the route of infection might be obtained by comparing the seroprevalences of different age-groups or groups of

animals which were housed together (Dijkstra et al., 2001a).

3.3.3.3. Serological examination of fetal body fluids. Because bovine fetuses develop immunocompetence around 120 gestational days (Swift and Kennedy, 1972), many but not all infected fetuses are able to develop specific antibodies to the transplacentally invading *N. caninum* tachyzoites (Bae et al., 2000; Barr et al., 1995; Bartley et al., 2004; Buxton et al., 1997; De Meerschman et al., 2002; Gondim et al., 2004b; Pereira-Bueno et al., 2003; Reichel and Drake, 1996; Slotved et al., 1999; Söndgen et al., 2001; Wouda et al., 1997a). This antibody response is a predominant IgG_1 response but also specific IgM and IgG_2 are detected (Andrianarivo et al., 2001; Buxton et al., 1997; Slotved et al., 1999).

Serological tests can be used to examine the infection status of an aborted fetus because fetal blood, serosanguinous fluids in the pleural or peritoneal body cavities, and abomasal content may contain specific antibodies against *N. caninum*. There are several reports indicating a low sensitivity when fetal serology is performed with IFAT or ELISA (Álvarez-García et al., 2003; Barr et al., 1995; Gottstein et al., 1998; Reichel and Drake, 1996; Slotved et al., 1999; Schock et al., 2000; Söndgen et al., 2001; Wouda et al., 1997a). The low sensitivity of fetal serology may be due to lack of fetal immunocompetence, especially in bovine fetuses younger than 6 months and a short interval between infection and fetal death (Wouda et al., 1997a). In addition, autolysis may cause degradation of fetal immunoglobulins (Wouda et al., 1997a) and may lead to low levels of specific antibodies. Thus, a negative serological result in an aborted fetus does not rule out *N. caninum* infection. Recently, Western blot-based assays are shown to increase sensitivity and specificity of fetal serology (Álvarez-García et al., 2002; Söndgen et al., 2001). However, it has to be stressed, that the demonstration of specific antibodies against *N. caninum* in an aborted fetus does not allow the conclusion that the parasite was responsible for disease because the vast majority of *N. caninum*-infected fetuses develop normally and are born as healthy calves (Paré et al., 1996). Nevertheless, demonstrating that a bovine fetus has developed antibodies against *N. caninum* is often the first specific indication for fetal infection during a diagnostic process (De Meerschman et al., 2002).

3.3.3.4. Examination of newborn calves. Fetal infection may lead to the birth of full-term congenitally infected calves that are clinically normal. However some calves may develop neurological disease (Barr et al., 1991b; De Meerschman et al., 2005; Dubey and De Lahunta, 1993; Dubey et al., 1990a). Intra-uterine infection with *N. caninum* seems to provoke the development of specific antibodies against the parasite in the majority of infected calves (Anderson et al., 1997; Ho et al., 1997a). Although pathogen specific tolerance in congenital *N. caninum* infection has been suspected (Anderson et al., 2000), the absence of antibodies to the parasite in stillborn or newborn calves makes a *N. caninum* infection unlikely. Newborn calves must be examined before suckling because colostral IgG antibodies (taken up via the gut) may cause false-positive test results (Jenkins et al., 1997; Paré et al., 1996). It has been shown that colostral antibodies in the calf persist for several months (Hietala and Thurmond, 1999; Wouda et al., 1998a).

Six cases of calves with neurological signs reported recently by De Meerschman et al. (2005) suggest a limited sensitivity of immunohistochemistry and PCR in neonatal bovine neosporosis but a reasonable performance of serological testing. However, as yet not enough data are available to suggest an appropriate cut-off in any of the serological tests meant to diagnose neosporosis in calves with neurological signs.

3.3.3.5. Serological tests to detect cattle infected with N. caninum. Different approaches to control bovine neosporosis on a herd level require sensitive and specific serological tests that are able to detect infected animals. For instance, identifying infected cattle may be necessary to prevent transmission of *N. caninum* during embryo transfer (Baillargeon et al., 2001; Landmann et al., 2002; Thurmond and Hietala, 1995). Other applications might aim to prevent the introduction of *N. caninum* into a herd through purchase of infected animals, or to assist culling of infected dams. As stated above, the majority of aborting dams has higher specific antibody levels compared to the majority of infected but non-aborting

dams (Dubey et al., 1997; McAllister et al., 1996a; Schares et al., 1999c, 2000; Waldner et al., 1998). The reliable detection of infected animals seems to require serological tests with a higher sensitivity than those meant to diagnose bovine abortion (Álvarez-García et al., 2003; Schares et al., 1999a). Since antibody levels in infected animals may fluctuate as a result of age (Davison et al., 1999a; Hietala and Thurmond, 1999) and the stage of gestation (see above) care must be taken when adjusting serological tests to this application of detecting infected animals. Recently, predominantly low *N. caninum*-specific antibody levels were reported for breeding bulls, suggesting that cut-offs optimised to detect infected breeding cows may be inappropriate to identify infected breeding bulls (Caetano-da-Silva et al., 2004a).

3.3.3.6. Serological tests to estimate the herd seroprevalence. Several working groups adapted ELISAs for analysis of bulk-milk samples (Table 2). An analysis with the ELISAs provides a roughly estimate of the seroprevalence within the group of animals that contributed to the bulk-milk sample (Bartels et al., 2005; Chanlun et al., 2002; Schares et al., 2003; Varcasia et al., 2006). As yet the sensitivity of these tests is limited: they detect herds with >10–20% seroprevalence. In addition to their application in epidemiological studies (Schares et al., 2003, 2004a), bulk-milk ELISAs are potentially important to support maintenance of bovine herds free of *N. caninum* infections and to identify herds already infected with this parasite (Bartels et al., 2005; Chanlun et al., 2006).

3.3.3.7. Serological tests to study epidemiology and control of neosporosis. Serological tests are important tools to examine the epidemiology of bovine neosporosis. The identification of vertical transmission as the predominant transmission route was largely based on serological results (Anderson et al., 1997). Seroepidemiological studies enabled to generate data on the importance of *N. caninum* as an abortifacient (e.g. Davison et al., 1999d; De Meerschman et al., 2000; Koiwai et al., 2005; Mainar-Jaime et al., 1999; Ould-Amrouche et al., 1999; Sager et al., 2001), its effect on other productivity parameters like reduced milk production (Hernandez et al., 2001; Hobson et al., 2002; Thurmond and Hietala, 1997; VanLeeuwen et al., 2001), premature culling (Cramer et al., 2002; Thurmond and Hietala, 1996; Tiwari et al., 2005; Waldner et al., 2001) and reduced weight gain (Barling et al., 2001; Waldner, 2002). In addition, the risk of abortion-infected cattle was estimated based on serological results (e.g. Hernandez et al., 2002; Thurmond and Hietala, 1997; Wouda et al., 1998a). Reactivation of chronic infection as a putative factor responsible for vertical transmission became likely when several independent studies observed an increase of specific antibody levels during gestation (Conrad et al., 1993b; Dannatt, 1998; Paré et al., 1997; Quintanilla-Gozalo et al., 2000; Stenlund et al., 1999). Further studies indicate that the kinetic of a serological response to *N. caninum* during pregnancy may help to predict abortion or vertical transmission in chronically infected dams (Guy et al., 2001; Paré et al., 1997; Pereira-Bueno et al., 2000).

By using the above-mentioned avidity ELISAs, recent infection was demonstrated to be the cause of abortion epidemics (Jenkins et al., 2000; McAllister et al., 2000; Sager et al., 2005; Schares et al., 2002). Although definitive proof is lacking, contaminations of fodder or drinking water by *N. caninum* oocysts are thought to be responsible for these infections (McAllister et al., 2000, 2005).

The putative role of dogs in the epidemiology of bovine neosporosis became evident by seroepidemiological studies (Bartels et al., 1999; Hobson et al., 2005; Mainar-Jaime et al., 1999; Ould-Amrouche et al., 1999; Paré et al., 1998; Schares et al., 2004a), one study (Paré et al., 1998) appearing at the same time it was shown that the dog is a definitive host of *N. caninum* (McAllister et al., 1998). The observation that in Texas, beef calves had an increased risk of seropositivity for *N. caninum* as a result of the abundance of wild canids, particularly coyotes and gray foxes (Barling et al., 2000) led to the hypothesis that a sylvatic transmission cycle for neosporosis exists (Barling et al., 2000). The recent finding that coyotes are also definitive hosts of *N. caninum*, confirmed the existence of a sylvatic life cycle (Gondim et al., 2004a).

3.4. Diagnosis by polymerase chain reaction (PCR)

The PCR plays an important role in the diagnosis of *N. caninum*-infection. Most PCR protocols are used to

detect *N. caninum* DNA in the body tissues of aborted fetuses or other intermediate hosts. However, also other samples like amniotic fluid (Ho et al., 1997a), cerebrospinal fluid (Buxton et al., 2001; Peters et al., 2000; Schatzberg et al., 2003), and oocyst contaminated dog or coyote feces (Basso et al., 2001; Gondim et al., 2004a; Hill et al., 2001; McGarry et al., 2003; Schares et al., 2005b; Šlapeta et al., 2002b) have been examined by PCR for the presence of *N. caninum* DNA. Initial attempts to detect *N. caninum* DNA in blood of naturally infected cattle failed (Guy et al., 2001). Recently, it was reported, that it is possible to identify *N. caninum* DNA in the blood of chronically infected cattle (Ferre et al., 2005; Okeoma et al., 2004a), in the milk of lactating cows (Moskwa et al., 2003) and in the semen of bulls (Caetano-da-Silva et al., 2004b; Ferre et al., 2005; Ortega-Mora et al., 2003).

Recently, PCR protocols were developed not only to detect but also to quantify *N. caninum* DNA in samples. Quantitative PCR has become one of the key-methodologies to examine the pathogenesis of bovine neosporosis and to assess the activity of vaccines and therapeutic or prophylactic drugs (Cannas et al., 2003a, 2003b; Collantes-Fernández et al., 2004, 2005; Esposito et al., 2005). Quantitative PCR was also used in epidemiological studies to estimate parasite load in *N. caninum* positive bull semen (Caetano-da-Silva et al., 2004b; Ferre et al., 2005; Ortega-Mora et al., 2003).

Different target DNAs were chosen to establish *N. caninum*-specific primer pairs. A repetitive character of the target DNA is an advantage since PCRs amplifying repetitive elements usually have a higher analytical sensitivity compared to PCRs amplifying fragments of single copy genes. Because of their repetitive character the genes coding for rRNA (see below) and the pNc5 gene (see below) have become important targets for diagnostic and quantitative PCRs.

The diagnostic sensitivity and the diagnostic specificity of a PCR is not only influenced by the selection of the appropriate target DNA and primer pairs, but also by selecting appropriate protocols to collect and to store samples, to extract and purify the sample DNA, to use appropriate reagents for PCR, to run the thermocycler, and to analyse the amplified DNA fragments (amplicons). It is important that those factors which may negatively influence the diagnostic sensitivity and specificity of a PCR are sufficiently controlled (Hoorfar et al., 2004). To observe poor quality DNA and to control false negatives due to inhibitory components in the template DNA, several *N. caninum* PCR protocols were developed which included internal controls such as the addition of a PCR MIMIC (Ellis et al., 1999b) or by using host DNA specific primers in a multiplex PCR (Baszler et al., 1999; Schatzberg et al., 2003; Meseck et al., 2005). Helpful PCR protocols are available in many text books or reviews and therefore will not be discussed here except for those aspects of specific importance for the application of PCR in the diagnosis of neosporosis.

A recent interlaboratory comparison on PCR diagnosis of *N. caninum* in bovine fetal tissues showed no clear relationship between the PCR format and the observed differences in diagnostic sensitivity and specificity between laboratories (Van Maanen et al., 2004). Consequently, prior to the application of the PCRs mentioned below to examine diagnostic samples, the entire process from the sample collection to analysis of the amplicons has to be validated (Conraths and Schares, 2006).

3.4.1. Sampling, sample-storage and DNA extraction

For diagnostic purposes and studies on the epidemiology of bovine neosporosis tissues of aborted fetuses, amniotic fluid, placenta, milk, fecal samples, environmental samples, fodder or water may be examined by PCR for the presence of *N. caninum* DNA.

In most studies on the examination of aborted fetuses or placenta by PCR, fresh samples from different tissues were collected and stored frozen (−20 °C) until used. Although it is well known that formaldehyde alters the quality of DNA significantly, successful examinations of sections of formaldehyde fixed and paraffin embedded tissues have been described (Baszler et al., 1999; Ellis et al., 1999b; Collantes-Fernández et al., 2002; Schatzberg et al., 2003). There is no general rule on which tissues are the most suitable for sampling. However, a number of studies indicate that brain tissue is the most suitable for the detection of *N. caninum* DNA by PCR followed by the heart, lung, and kidneys (Baszler et al., 1999; Buxton et al., 1998; Collantes-Fernández et al., 2005; Gottstein et al., 1998; Ho et al.,

1997a). The parasite load may be influenced by the stage of gestation at which the fetus was aborted (Collantes-Fernández et al., 2005). In the last trimester, the parasite was only detected in the brain and, sporadically, in the diaphragm, heart and lymph nodes (Collantes-Fernández et al., 2005). There are reports that *N. caninum* DNA was amplified from amniotic fluid and placenta (Buxton et al., 1998; Gottstein et al., 1998; Ho et al., 1997a).

Fetal tissues and placenta that are submitted for examination may be in different stages of autolysis. There are no data on how autolysis influences the diagnostic sensitivity of *N. caninum*-specific PCRs. It was assumed that specific PCRs based the amplification of small fragments are less affected by autolytic processes than PCRs based on larger amplicons (Ellis et al., 1999b).

Dogs and coyotes are definitive hosts of *N. caninum* (Gondim et al., 2004a; Lindsay et al., 1999a, 1999b, 2001; McAllister et al., 1998). Canine feces or environmental samples contaminated by dog feces may contain *N. caninum* oocysts. DNA extracted from feces or environmental samples are known to contain PCR inhibitors. However, only sparse information is available on the best way to enrich and purify *N. caninum* DNA from such samples (Basso et al., 2001; Gondim et al., 2004a; Hill et al., 2001; McGarry et al., 2003; Schares et al., 2005a, 2005b; Šlapeta et al., 2002b). Protocols developed for *Hammondia heydorni* (Ellis et al., 1999a; Šlapeta et al., 2002a) may help to establish suitable techniques to examine feces or environmental samples for *N. caninum*. Methods used to detect *T. gondii* oocysts in soil or water may also be applicable after modification (Dumètre and Dardé, 2003, 2005).

3.4.2. Target genes used for the establishment of diagnostic *N. caninum* PCRs

Using isolates like NC-1, NC-2, NC-3, Nc-LIV, BPA-1, Nc-SweB1, a number of PCRs were developed to specifically amplify *N. caninum* DNA (Table 4). Since the genomic sequences of DNA coding for ribosomal RNA (rDNA) can be used for phylogenetic studies among related apicomplexan species (e.g. *T. gondii*, *N. caninum*, *H. hammondi* and *H. heydorni*) rDNA sequences (18S rDNA, 28S rDNA, ITS1) are promising targets for the development of species specific PCRs. Other targets include the pNc5 gene, a multicopy gene (Müller et al., 2001), and a singlecopy gene, 14–3–3 (Lally et al., 1996a).

3.4.2.1. The 18S rDNA. Only minor differences have been found between the 18S rDNA genes of *T. gondii* and *N. caninum* suitable for the development of species specific primers (Marsh et al., 1995). Therefore, universal primers had to be used to amplify the 18S rDNA (Ellis, 1998; Ho et al., 1996; Magnino et al., 1999). In one study, the application of species specific chemiluminescent DNA probes allowed differentiation of *Neospora* and *Toxoplasma* 18S rDNA amplicons (Ho et al., 1996). This protocol was applied in a subsequent study on the distribution of *N. caninum* DNA in bovine tissues (Ho et al., 1997a), but also in a study to characterize a bovine *N. caninum* isolate (Kim et al., 2000). It was used further to demonstrate vertical transmission in rhesus macaques (Ho et al., 1997b). Another protocol, developed by Ellis (1998) also used universal primers to amplify 18S rDNA in the first round PCR. In a second round, a *N. caninum*-specific primer (containing two mismatches) was used to differentiate *N. caninum* 18S rDNA from that of *Toxoplasma*, *S. cruzi*, and host cell DNA (Ellis, 1998). A third protocol was developed by Magnino et al. (1999) which applied the slightly modified primer pair developed by Ho et al. (1996), but differentiated *Neospora*, *Toxoplasma* and *Sarcocystis* 18S rDNA by differences in fragmentation using the restriction enzyme BsaJI. Recently, it was demonstrated that also *Hammondia* 18S rDNA is amplified by these universal primers and specific fragments are obtained after using SecI, an isoenzyme of BsaJI (Schares et al., 2005b). This protocol has the advantage that the DNA of different species are amplified simultaneous in a single reaction, i.e. the results for different species are obtained by a method exhibiting the same sensitivity (Eleni et al., 2004; Schares et al., 2005b; Van Maanen et al., 2004).

3.4.2.2. The 28S rDNA. The D2 domain of the 28S rDNA is also used for phylogenetic studies. Based on the species specific sequences, Ellis et al. (1998) identified an *N. caninum*-specific primer pair which was able to distinguish *N. caninum* from *Toxoplasma*, *Hammondia* or *Besnoitia*. Yet, no data on the analytical sensitivity of this PCR are available.

Table 4
Analytical sensitivity and specificity of polymerase chain reactions for the detection *N. caninum* DNA

Target DNA	Primer names	Type of PCR	Analytical sensitivity according to the original description	Parasites used to test analytical specificity	Reference
18S rDNA	COC-1, COC-2	One-step PCR + hybridisation	1 tachyzoite in medium, 5 tachyzoites in blood or amniotic fluid	*T. gondii*, *S. cruzi*, *S. tenella*, *S. capracanis*, *C. parvum*, *E. bovis*	Ho et al. (1996)
18S rDNA	COC-1, COC-2	One-step PCR + restriction enzyme	ND	*T. gondii*	Magnino et al. (1999)
18S rDNA	AP1, D SP4, A	Two-step nested PCR	ND	*T. gondii*, *S. cruzi*	Ellis (1998)
28S rDNA	GA1, NF6	One-step PCR	ND	*T. gondii*, *H. hammondi*, *B. besnoiti*	Ellis et al. (1998)
ITS1	NS1, SR1	One-step PCR	ND	*T. gondii*, *S. cruzi*	Payne and Ellis (1996)
ITS1	PN1, PN2	One-step PCR	5 tachyzoites heated 2 min at 100 °C in distilled water	*T. gondii*, *S. cruzi*, *S. fusiformis*, *S. gigantea*, *S. tenella*	Holmdahl and Mattsson (1996)
ITS1	NN1, NN2 NP1, NP2	Two-step nested PCR	ND	ND	Buxton et al. (1998)
ITS1	TIM3, TIM11 NS1, SR1	Two-step nested PCR	ND	See Payne and Ellis (1996)	Ellis (1998)
ITS1	F6, 5.8B PN3, PN4	Two-step nested PCR	ND[a]	ND	Uggla et al. (1998)
ITS1	NS2, NR1, NF1, SR1	One-step nested PCR	10–1 fg DNA (0.1–0.01 tachyzoites)	*T. gondii*, *S. cruzi*	Ellis et al. (1999a, 1999b)
pNc5 gene	Np1, Np 2	One-step PCR	100 pg genomic tachyzoite DNA	*T. gondii*, *S. cruzi*, *S. ovifelis*, *S. capracanis*, *S. moulei*, *S. miescheriana*	Kaufmann et al. (1996)
pNc5 gene	Np6, Np21	One-step PCR	1 tachyzoite in 1 mg brain tissue	*T. gondii*, *H. hammondi*, *S. cruzi*, *S. tenella*, *S. capracanis*, *S. moulei*, *S. miescheriana*, *H. heydorni*[b], *Toxocara canis*[b]	Yamage et al. (1996)
pNc5 gene	Np6plus, Np21plus	One-step PCR	DNA equivalent to 1–10 tachyzoite genomes	*T. gondii*, *H. hammondi*, *S. cruzi*, *S. tenella*, *S. capracanis*, *S. moulei*, *S. miescheriana*	Müller et al. (1996)
pNc5 gene	Np6plus, Np21plus	One-step PCR + hybridisation	DNA equivalent to 1 tachyzoite genomes	*T. gondii*, *H. hammondi*, *S. cruzi*, *S. tenella*, *S. capracanis*, *S. moulei*, *S. miescheriana*	Müller et al. (1996)
pNc5 gene	Np4, Np7	One-step PCR	1–2 tachyzoite equivalents per DNA sample (150 ng brain tissue DNA)	See Yamage et al. (1996)	Baszler et al. (1999)

pNc5 gene	Np4, Np7 Np6, Np7	Two-step seminested PCR	ND; sensitivity of seminested PCR was not superior to one-step Np6–Np7 PCR	See Yamage et al. (1996)	Baszler et al. (1999)
pNc5 gene	Np6plus, Np21plus	One-step quantitative PCR	9 fg DNA per 250 ng of mouse DNA	See Müller et al. (1996)	Liddell et al. (1999a, 1999b)
pNc5 gene	Np6plus, Np21plus	Real-time PCR (probes)	DNA equivalent to 1 tachyzoite	See Müller et al. (1996)	Müller et al. (2001)
pNc5 gene	Np4B, Np21B	One-step PCR	ND	See Yamage et al. (1996)	Bergeron et al. (2001)
pNc5 gene	Nc5fwd, Nc5rev	Real-time PCR (Cyber green)	DNA equivalent to 0.1 tachyzoite genomes (10 fg) in 100 ng mouse brain DNA	*T. gondii*	Collantes-Fernández et al. (2002)
14–3–3 gene	Nc13F3, Nc13R2 Nc13F1, Nc13R4	Two-step nested PCR	25 tachyzoites in 5 mg brain tissue	*T. gondii*, *S. muris*, *S. tenella*, *S. cruzi*	Lally et al. (1996a, 1996b)

ND, no data.
[a] Some information on analytical sensitivity in Guy et al. (2001).
[b] Analysed by Hill et al. (2001).

3.4.2.3. The internal transcribed spacer 1 (ITS1) region of the rRNA gene. Among the ITS1 regions of *T. gondii*, *N. caninum*, *H. heydorni* and *H. hammondi* there are a number of sequence differences that allow the establishment of species specific PCRs. Many PCR protocols are published using the ITS1 region as the target. In addition to single-step conventional PCRs (Holmdahl and Mattsson, 1996), two-step nested PCRs were developed (Buxton et al., 1998; Uggla et al., 1998) and used in studies on the epidemiology and pathogenesis of bovine neosporosis (Buxton et al., 2001; Caetano-da-Silva et al., 2004b; Collantes-Fernández et al., 2002, 2005; Ferre et al., 2005; Guy et al., 2001; McGarry et al., 2003; Ortega-Mora et al., 2003; Pereira-Bueno et al., 2003; Serrano et al., 2006; Trees et al., 2002; Van Maanen et al., 2004; Williams et al., 2000).

Although two-step nested PCRs usually are superior in sensitivity, they have the disadvantage of having a higher risk of carryover contaminations. An alternative are single-step (single-tube) nested PCRs which combine the higher sensitivity of a nested PCR with the lower risk of carryover contaminations in single-step PCRs. Ellis et al. (1999b) developed a ITS1-based one-tube nested PCR for *N. caninum*. An analytical sensitivity of 1–10 fg genomic DNA of *N. caninum* tachyzoites was reported for this protocol. One to 10 fg DNA is equivalent to the genomic DNA of 0.1–0.01 tachyzoites. This is the highest analytical sensitivity so far reported for a specific *N. caninum* PCR. This PCR was recently used to examine aborted fetuses in Mexico (Medina et al., 2006). Williams et al. (2000), Davison et al. (2001) and McGarry et al. (2003) used the primers of Ellis et al. (1999b) but in a two-step nested PCR.

3.4.2.4. The pNc5 gene. Kaufmann et al. (1996) and Yamage et al. (1996) identified the obviously repetitive DNA sequence in the *N. caninum* genome (Müller et al., 2001). The corresponding gene – the pNc5 gene – seems not to exist in the genome of *T. gondii*, *S. cruzi*, or *H. hammondi* although cross-reactivity with *S. cruzi* was reported for one of the evaluated primer pairs (Np5, Np6; Yamage et al., 1996). Initially, several primer pairs were developed (Np1 to Np8, and Np21). However, in addition to the primer pair Np1 and Np2 (Kaufmann et al., 1996) the pair Np6 and Np21 appeared to be the most suitable one (Yamage et al., 1996). A conventional single-step

PCR using these primers was able to detect one tachyzoite in 2 mg brain tissue. This PCR has been used in many studies (Basso et al., 2001; Dreier et al., 1999; Edelhofer et al., 2003; Gondim et al., 2001, 2004a; Hill et al., 2001; Huang et al., 2004a, 2004b; Kobayashi et al., 2001; Koyama et al., 2001; Lemberger et al., 2005; McAllister et al., 1998, 1999; McGuire et al., 1999; Meseck et al., 2005; Moskwa et al., 2003; Rodrigues et al., 2004; Sawada et al., 2000; Schatzberg et al., 2003; Spencer et al., 2000; Sreekumar et al., 2003, 2004). Recently the primers Np6 and Np21 were also used to establish an in situ PCR to detect *N. caninum* DNA in histological sections (Löschenberger et al., 2004).

Some working groups used the primers Np4, Np6 and NP7 to detect the pNc5 target (Baszler et al., 1999; Kim et al., 2002; Rettigner et al., 2004; Soldati et al., 2004). Others have modified published primers to amplify fragments of the pNc5 gene (Bergeron et al., 2001) and have developed their own primers (Collantes-Fernández et al., 2002) which were used in a quantitative PCR (see also Caetano-da-Silva et al., 2004a, 2004b; Ferre et al., 2005; Ortega-Mora et al., 2003). Müller et al. (1996) developed a single-step PCR using slightly modified primers Np6plus and Np21plus. The amplicons were detected using a hybridisation ELISA. This PCR method was able to detect one tachyzoite in 1 mg tissue. Without hybridisation, but using the modified primers, the PCR had a sensitivity of 1–10 tachyzoites in 1 mg tissue. The primer pair Np6plus and Np21plus have been used by the majority of studies were a *N. caninum* PCR was applied (Almería et al., 2002; Ammann et al., 2004; Dijkstra et al., 2001b; Dubey et al., 1998b, 2004; Eperon et al., 1999; Gottstein et al., 1998, 1999, 2001; Hässig and Gottstein, 2002; Hässig et al., 2003; Henning et al., 2002; Hůrková and Modrý, 2006; Liddell et al., 1999a, 1999b, 1999c; McGarry et al., 2003; Müller et al., 2001; Okeoma et al., 2004a; Peters et al., 2000, 2001b; Pitel et al., 2002; Reichel et al., 1998; Sager et al., 2001; Šlapeta et al., 2002b; Söndgen et al., 2001; Schares et al., 1997; Schares et al., 2005b; Van Maanen et al., 2004). Recently, the primers Np6plus and Np21plus (Müller et al., 2001) were used in combination with the primers Np6 and Np7 (Baszler et al., 1999; Yamage et al., 1996) in a two-step nested PCR approach to detect natural infections with *N. caninum* in mice, rats and sheep (Hughes et al., 2006).

3.4.2.5. The 14–3–3 gene. Lally et al. (1996a) developed a two-step nested PCR based on the 14–3–3 gene. Although this gene is evolutionarily conserved among eukaryotic taxa it was possible to identify primers that proofed to by *N. caninum*-specific using DNA from *T. gondii*, *S. cruzi*, *S. tenella* and *S. muris*. This PCR was shown to be able to detect 25 *N. caninum* tachyzoites in 5 mg brain tissue. Dubey et al. (1998b) applied this PCR to examine tissues of a naturally infected dog.

3.4.3. Analytical specificity of PCRs developed to detect N. caninum

For the majority of PCR protocols developed to detect *N. caninum* DNA, it was shown that no amplification products occur when the DNA of *T. gondii* or *S. cruzi* is examined, which are both parasites that may occur in tissues of infected intermediate hosts (e.g. bovine fetuses) or environmental samples (Table 4). Some of the PCR protocols are further evaluated for their specificity using DNA from further *Sarcocystis* sp., *Eimeria bovis*, *Besnoitia besnoiti*, *Hammondia hammondi* and *H. heydorni*. In one study DNA from bacterial pathogens was used to demonstrate specificity (Ho et al., 1996).

Most diagnostic *Neospora*-PCRs were developed prior to the description of another *Neospora* species, *N. hughesi* (Marsh et al., 1998). It was not known if primers developed for the diagnosis of *N. caninum* would amplify *N. hughesi* DNA. Sequence information is now available on the ITS1 and the 28S rDNA of *N. hughesi*. When this sequence information is compared with the published primer sequences for *N. caninum* it becomes obvious that in all the available PCR protocols reviewed in this paper, using ITS1 or 28S rDNA as a target, at least one primer has sequence differences in at least one base-pair (Ellis et al., 1999a; Schares, unpublished). It may be that these ITS1 and 28S rDNA based PCRs for *N. caninum* either do not detect *N. hughesi* DNA or with reduced sensitivity. Prior to the use of these primer pairs to detect *N. hughesi* DNA, an evaluation of their sensitivity is necessary. The amplification of the pNc5 gene fragments by Np6 and Np21 could not be demonstrated using *N. hughesi* DNA (Basso et al., 2001; Spencer et al., 2000). Therefore, it is very likely that also the PCR using the modified primers Np6plus and Np21plus (Müller et al., 1996) are *N. caninum*-specific

and do not amplify *N. hughesi* DNA. However, other primer pairs also developed to amplify pNc5 gene fragments (Baszler et al., 1999; Bergeron et al., 2001; Collantes-Fernández et al., 2002; Kaufmann et al., 1996; Yamage et al., 1996) have not yet examined for the amplification of *N. hughesi* DNA. Interestingly, no *N. hughesi*-specific PCR is currently available.

3.4.4. Quantitative PCR

Quantitative PCRs are important tools to examine the pathogenesis of bovine neosporosis and to assess the activity of vaccines and therapeutic or prophylactic drugs. Conventional single- or two-step PCRs are non-quantitative but qualitative detection methods. All quantitative PCRs, published as yet, are based on the pNc5 gene. A first quantitative PCR was established as a so called quantitative-competitive PCR (QC-PCR; Liddell et al., 1999b; Van Maanen et al., 2004). *N. caninum*-specific DNA is amplified in the presence of an artificial competitor and examined in ethidium bromide gels. Competitor titration allows the estimation of copies of pNc5 gene per sample. This method is labour intensive compared to real-time PCR approaches (Collantes-Fernández et al., 2002; Müller et al., 2002; Van Maanen et al., 2004). One real-time PCR is based on detection of PCR products by specific fluorescent probes (Müller et al., 2002) and was used to demonstrate development of infection in an organotypic slice culture system for *N. caninum* (Vonlaufen et al., 2002), for assessing vaccination or drug trials (Cannas et al., 2003a, 2003b; Esposito et al., 2005), and to study the cell biology of *N. caninum* (Naguleswaran et al., 2003). Another approach employs the double-strand DNA-binding dye SYBR Green I (Collantes-Fernández et al., 2002). By using the SYBR Green I based real-time PCR the amount of tachyzoites in the brain samples of aborted fetuses was shown to range between 2.9 and 26.6 mg^{-1} brain. This methodology was also used recently to estimate the parasite load in various tissues of fetuses aborted at different stages of gestation (Collantes-Fernández et al., 2005).

Acknowledgements

We would like to thank Drs. David Buxton, John Ellis, Dolores Hill and Willem Wouda for their help.

References

Aguado-Martinez, A., Álvarez-Garcia, G., Arnaiz-Seco, I., Innes, E., Ortega-Mora, L.M., 2005. Use of avidity enzyme-linked immunosorbent assay and avidity Western blot to discriminate between acute and chronic *Neospora caninum* infection in cattle. J. Vet. Diagn. Invest. 17, 442–450.

Ahn, H.J., Kim, S., Kim, D.Y., Nam, H.W., 2003. ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. Korean J. Parasitol. 41, 175–177.

Almería, S., Ferrer, D., Pabón, M., Castellà, J., Mañas, S., 2002. Red foxes (*Vulpes vulpes*) are a natural intermediate host of *Neospora caninum*. Vet. Parasitol. 107, 287–294.

Álvarez-García, G., Pereira-Bueno, J., Gómez-Bautista, M., Ortega-Mora, L.M., 2002. Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Vet. Parasitol. 107, 15–27.

Álvarez-García, G., Collantes-Fernández, E., Costas, E., Rebordosa, X., Ortega-Mora, L.M., 2003. Influence of age and purpose for testing on the cut-off selection of serological methods in bovine neosporosis. Vet. Res. 34, 341–352.

Ammann, P., Waldvogel, A., Breyer, I., Esposito, M., Müller, N., Gottstein, B., 2004. The role of B- and T-cell immunity in toltrauril-treated C57BL/6 WT, μMT and nude mice experimentally infected with *Neospora caninum*. Parasitol. Res. 93, 178–187.

Anderson, M.L., Blanchard, P.C., Barr, B.C., Dubey, J.P., Hoffman, R.L., Conrad, P.A., 1991. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. J. Am. Vet. Med. Assoc. 198, 241–244.

Anderson, M.L., Palmer, C.W., Thurmond, M.C., Picanso, J.P., Blanchard, P.C., Breitmeyer, R.E., Layton, A.W., McAllister, M., Daft, B., Kinde, H., Read, D.H., Dubey, J.P., Conrad, P.A., Barr, B.C., 1995. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. J. Am. Vet. Med. Assoc. 207, 1206–1210.

Anderson, M.L., Reynolds, J.P., Rowe, J.D., Sverlow, K.W., Packham, A.E., Barr, B.C., Conrad, P.A., 1997. Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. J. Am. Vet. Med. Assoc. 210, 1169–1172.

Anderson, M.L., Andrianarivo, A.G., Conrad, P.A., 2000. Neosporosis in cattle. Anim. Reprod. Sci. 60–61, 417–431.

Andrianarivo, A.G., Barr, B.C., Anderson, M.L., Rowe, J.D., Packham, A.E., Sverlow, K.W., Conrad, P.A., 2001. Immune responses in pregnant cattle and bovine fetuses following experimental infection with *Neospora caninum*. Parasitol. Res. 87, 817–825.

Atkinson, R.A., Cook, R.W., Reddacliff, L.A., Rothwell, J., Broady, K.W., Harper, P.A.W., Ellis, J.T., 2000. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. Aust. Vet. J. 78, 262–266.

Bae, J.S., Kim, D.Y., Hwang, W.S., Kim, J.H., Lee, N.S., Nam, H.W., 2000. Detection of IgG antibody against *Neospora caninum* in cattle in Korea. Korean J. Parasitol. 38, 245–249.

Baillargeon, P., Fecteau, G., Paré, J., Lamothe, P., Sauvé, R., 2001. Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of control-

ling vertical transmission of *Neospora caninum* in cattle. J. Am. Vet. Med. Assoc. 218, 1803–1806.

Barling, K.S., Sherman, M., Peterson, M.J., Thompson, J.A., McNeill, J.W., Craig, T.M., Adams, L.G., 2000. Spatial associations among density of cattle, abundance of wild canids, and seroprevalence to *Neospora caninum* in a population of beef calves. J. Am. Vet. Med. Assoc. 217, 1361–1365.

Barling, K.S., Lunt, D.K., Snowden, K.F., Thompson, J.A., 2001. Association of serologic status for *Neospora caninum* and postweaning feed efficiency in beef steers. J. Am. Vet. Med. Assoc. 219, 1259–1262.

Barr, B.C., Anderson, M.L., Blanchard, P.C., Daft, B.M., Kinde, H., Conrad, P.A., 1990. Bovine fetal encephalitis and myocarditis associated with protozoal infections. Vet. Pathol. 27, 354–361.

Barr, B.C., Anderson, M.L., Dubey, J.P., Conrad, P.A., 1991a. *Neospora*-like protozoal infections associated with bovine abortions. Vet. Pathol. 28, 110–116.

Barr, B.C., Conrad, P.A., Dubey, J.P., Anderson, M.L., 1991b. *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. J. Vet. Diagn. Invest. 3, 39–46.

Barr, B.C., Conrad, P.A., Breitmeyer, R., Sverlow, K., Anderson, M.L., Reynolds, J., Chauvet, A.E., Dubey, J.P., Ardans, A.A., 1993. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*-infected fetuses: four cases (1990–1992). J. Am. Vet. Med. Assoc. 202, 113–117.

Barr, B.C., Anderson, M.L., Sverlow, K.W., Conrad, P.A., 1995. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test. Vet. Rec. 137, 611–613.

Barta, J.R., Dubey, J.P., 1992. Characterization of anti-*Neospora caninum* hyperimmune rabbit serum by Western blot analysis and immunoelectron microscopy. Parasitol. Res. 78, 689–694.

Bartels, C.J., Wouda, W., Schukken, Y.H., 1999. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in the Netherlands (1995–1997). Theriogenology 52, 247–257.

Bartels, C.J.M., van Maanen, C., van der Meulen, A.M., Dijkstra, T., Wouda, W., 2005. Evaluation of three enzyme-linked immunosorbent assays for detection of antibodies to *Neospora caninum* in bulk milk. Vet. Parasitol. 131, 235–246.

Bartley, P.M., Kirvar, E., Wright, S., Swales, C., Esteban-Redondo, I., Buxton, D., Maley, S.W., Schock, A., Rae, A.G., Hamilton, C., Innes, E.A., 2004. Maternal and fetal immune responses of cattle inoculated with *Neospora caninum* at mid-gestation. J. Comp. Pathol. 130, 81–91.

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C.H., Shen, S.K., Dubey, J.P., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. J. Parasitol. 87, 612–618.

Baszler, T.V., Knowles, D.P., Dubey, J.P., Gay, J.M., Mathison, B.A., McElwain, T.F., 1996. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol. 34, 1423–1428.

Baszler, T.V., Gay, L.J.C., Long, M.T., Mathison, B.A., 1999. Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. J. Clin. Microbiol. 37, 4059–4064.

Baszler, T.V., Adams, S., Vander-Schalie, J., Mathison, B.A., Kostovic, M., 2001. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. J. Clin. Microbiol. 39, 3851–3857.

Bergeron, N., Girard, C., Paré, J., Fecteau, G., Robinson, J., Baillargeon, P., 2001. Rare detection of *Neospora caninum* in placentas from seropositive dams giving birth to full-term calves. J. Vet. Diagn. Invest. 13, 173–175.

Bjerkås, I., Jenkins, M.C., Dubey, J.P., 1994. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. Clin. Diagn. Lab. Immunol. 1, 214–221.

Björkman, C., Lundén, A., Holmdahl, J., Barber, J., Trees, A.J., Uggla, A., 1994. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. Parasite Immunol. 16, 643–648.

Björkman, C., Holmdahl, O.J.M., Uggla, A., 1997. An indirect enzyme-linked immumoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. Vet. Parasitol. 68, 251–260.

Björkman, C., Hemphill, A., 1998. Characterization of *Neospora caninum* iscom antigens using monoclonal antibodies. Parasite Immunol. 20, 73–80.

Björkman, C., Näslund, K., Stenlund, S., Maley, S.W., Buxton, D., Uggla, A., 1999. An IgG avidity ELISA to discriminate between recent and chronic *Neospora caninum* infection. J. Vet. Diagn. Invest. 11, 41–44.

Björkman, C., McAllister, M.M., Frössling, J., Näslund, K., Leung, F., Uggla, A., 2003. Application of the *Neospora caninum* IgG avidity ELISA in assessment of chronic reproductive losses after an outbreak of neosporosis in a herd of beef cattle. J. Vet. Diagn. Invest. 15, 3–7.

Björkman, C., Gondim, L.F.P., Näslund, K., Trees, A.J., McAllister, M.M., 2005. IgG avidity pattern in cattle after ingestion of *Neospora caninum* oocysts. Vet. Parasitol. 128, 195–200.

Boger, L.A., Hattel, A.L., 2003. Additional evaluation of undiagnosed bovine abortion cases may reveal fetal neosporosis. Vet. Parasitol. 113, 1–6.

Boulton, J.G., Gill, P.A., Cook, R.W., Fraser, G.C., Harper, P.A.W., Dubey, J.P., 1995. Bovine *Neospora* abortion in north-eastern New South Wales. Aust. Vet. J. 72, 119–120.

Buxton, D., Caldow, G.L., Maley, S.W., Marks, J., Innes, E.A., 1997. Neosporosis and bovine abortion in Scotland. Vet. Rec. 141, 649–651.

Buxton, D., Maley, S.W., Wright, S., Thomson, K.M., Rae, A.G., Innes, E.A., 1998. The pathogenesis of experimental neosporosis in pregnant sheep. J. Comp. Pathol. 118, 267–279.

Buxton, D., Wright, S., Maley, S.W., Rae, A.G., Lundén, A., Innes, E.A., 2001. Immunity to experimental neosporosis in pregnant sheep. Parasite Immunol. 23, 85–91.

Caetano-da-Silva, A., Ferre, I., Aduriz, G., Álvarez-García, G., del Pozo, I., Atxaerandio, R., Regidor-Cerrillo, J., Ugarte-Garagalza, C., Ortega-Mora, L.M., 2004a. *Neospora caninum* infection in breeder bulls: seroprevalence and comparison of serological methods used for diagnosis. Vet. Parasitol. 124, 19–24.

Caetano-da-Silva, A., Ferre, I., Collantes-Fernández, E., Navarro, V., Aduriz, G., Ugarte-Garagalza, C., Ortega-Mora, L.M., 2004b. Occasional detection of *Neospora caninum* DNA in frozen extended semen from naturally infected bulls. Theriogenology 62, 1329–1336.

Canada, N., Meireles, C.S., Rocha, A., Sousa, S., Thompson, G., Dubey, J.P., Romand, S., Thulliez, P., Correia da Costa, J.M., 2002a. First Portuguese isolate of *Neospora caninum* from an aborted fetus from a dairy herd with endemic neosporosis. Vet. Parasitol. 110, 11–15.

Canada, N., Meireles, C.S., Rocha, A., Correia da Costa, J.M., Erickson, M.W., Dubey, J.P., 2002b. Isolation of viable *Toxoplasma gondii* from naturally-infected aborted bovine fetuses. J. Parasitol. 88, 1247–1248.

Canada, N., Meireles, C.S., Carvalheira, J., Rocha, A., Sousa, S., Correia da Costa, J.M., 2004a. Determination of an optimized cut-off value for the *Neospora* agglutination test for serodiagnosis in cattle. Vet. Parasitol. 121, 225–231.

Canada, N., Meireles, C.S., Mezo, M., González-Warleta, M., Correia da Costa, J.M., Sreekumar, C., Hill, D.E., Miska, K.B., Dubey, J.P., 2004b. First isolation of *Neospora caninum* from an aborted bovine fetus in Spain. J. Parasitol. 90, 863–864.

Cannas, A., Naguleswaran, A., Muller, N., Eperon, S., Gottstein, B., Hemphill, A., 2003a. Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology 126, 303–312.

Cannas, A., Naguleswaran, A., Müller, N., Gottstein, B., Hemphill, A., 2003b. Reduced cerebral infection of *Neospora caninum*-infected mice after vaccination with recombinant microneme protein NcMIC3 and RIBI adjuvant. J. Parasitol. 89, 44–50.

Chahan, B., Gaturaga, I., Huang, X., Liao, M., Fukumoto, S., Hirata, H., Nishikawa, Y., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K., Igarashi, I., Mikami, T., Xuan, X., 2003. Serodiagnosis of *Neospora caninum* infection in cattle by enzyme-linked immunosorbent assay with recombinant truncated NcSAG1. Vet. Parasitol. 118, 177–185.

Chanlun, A., Näslund, K., Aiumlamai, S., Björkman, C., 2002. Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand. Vet. Parasitol. 110, 35–44.

Chanlun, A., Emanuelson, U., Chanlun, S., Aiumlamai, S., Björkman, C., 2006. Application of repeated bulk milk testing for identification of infection dynamics of *Neospora caninum* in Thai dairy herds. Vet. Parasitol. 136, 243–250.

Cheah, T.S., Mattsson, J.G., Zaini, M., Sani, R.A., Jakubek, E.B., Uggla, A., Chandrawathani, P., 2004. Isolation of *Neospora caninum* from a calf in Malaysia. Vet. Parasitol. 126, 263–269.

Choromanski, L., Block, W., 2000. Humoral immune responses and safety of experimental formulations of inactivated *Neospora* vaccines. Parasitol. Res. 86, 851–853.

Cole, R.A., Lindsay, D.S., Dubey, J.P., Toivio-Kinnucan, M.A., Blagburn, B.L., 1994. Characterization of a murine monoclonal antibody generated against *Neospora caninum* by western blot analysis and immunoelectron microscopy. Am. J. Vet. Res. 55, 1717–1722.

Collantes-Fernández, E., Zaballos, Á., Álvarez-García, G., Ortega-Mora, L.M., 2002. Quantitative detection of *Neospora caninum* in bovine aborted fetuses and experimentally infected mice by real-time PCR. J. Clin. Microbiol. 40, 1194–1198.

Collantes-Fernández, E., Álvarez-García, G., Pérez-Pérez, V., Pereira-Bueno, J., Ortega-Mora, L.M., 2004. Characterization of pathology and parasite load in outbred and inbred mouse models of chronic *Neospora caninum* infection. J. Parasitol. 90, 579–583.

Collantes-Fernández, E., Rodriguez-Bertos, A., Arnaiz-Seco, I., Moreno, B., Aduriz, G., Ortega-Mora, L.M., 2005. Influence of the stage of pregnancy on *Neospora caninum* distribution, parasite loads and lesions in aborted bovine foetuses. Theriogenology 65, 629–641.

Conrad, P.A., Barr, B.C., Sverlow, K.W., Anderson, M., Daft, B., Kinde, H., Dubey, J.P., Munson, L., Ardans, A., 1993a. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology 106, 239–249.

Conrad, P.A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Palmer, C., Thurmond, M., Ardans, A., Dubey, J.P., Duhamel, G., Barr, B., 1993b. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. J. Vet. Diagn. Invest. 5, 572–578.

Conraths, F.J., Ortega, L.M., 2005. Workshop conclusions: options for control of protozoal abortion in ruminants—practical experience. In: Proceedings of the 20th International Conference of World Association for the Advancement of Veterinary Parasitology, vol. 20. supplementary pages.

Conraths, F.J., Schares, G., 2006. Validation of molecular-diagnostic techniques in the parasitological laboratory. Vet. Parasitol. 136, 91–98.

Cornelissen, A.W.C.A., Overdulve, J.P., Hoenderboom, J.M., 1981. Separation of *Isospora* (*Toxoplasma*) *gondii* cysts and cystozoites from mouse brain tissue by continuous density-gradient centrifugation. Parasitology 83, 103–108.

Cramer, G., Kelton, D., Duffield, T.F., Hobson, J.C., Lissemore, K., Hietala, S.K., Peregrine, A.S., 2002. *Neospora caninum* serostatus and culling of Holstein cattle. J. Am. Vet. Med. Assoc. 221, 1165–1168.

Cuddon, P., Lin, D.S., Bowman, D.D., Lindsay, D.S., Miller, T.K., Duncan, I.D., DeLahunta, A., Cummings, J., Suter, M., Cooper, B., King, J.M., Dubey, J.P., 1992. *Neospora caninum* infection in English springer spaniel littermates: diagnostic evaluation and organism isolation. J. Vet. Intern. Med. 6, 325–332.

Dannatt, L., 1998. *Neospora caninum* antibody levels in an endemically-infected dairy herd. Irish Vet. J. 51, 200–201 (reprinted from Cattle Practice 5, 335–337).

Davison, H.C., French, N.P., Trees, A.J., 1999a. Herd-specific and age-specific seroprevalence of *Neospora caninum* in 14 British dairy herds. Vet. Rec. 144, 547–550.

Davison, H.C., Guy, F., Trees, A.J., Ryce, C., Ellis, J.T., Otter, A., Jeffrey, M., Simpson, V.R., Holt, J.J., 1999b. In vitro isolation of *Neospora caninum* from a stillborn calf in the UK. Res. Vet. Sci. 67, 103–105.

Davison, H.C., Otter, A., Trees, A.J., 1999c. Estimation of vertical and horizontal transmission parameters of *Neospora*

caninum infections in dairy cattle. Int. J. Parasitol. 29, 1683–1689.

Davison, H.C., Otter, A., Trees, A.J., 1999d. Significance of *Neospora caninum* in British dairy cattle determined by estimation of seroprevalence in normally calving cattle and aborting cattle. Int. J. Parasitol. 29, 1189–1194.

Davison, H.C., Guy, C.S., McGarry, J.W., Guy, F., Williams, D.J.L., Kelly, D.F., Trees, A.J., 2001. Experimental studies on the transmission of *Neospora caninum* between cattle. Res. Vet. Sci. 70, 163–168.

De Marez, T., Liddell, S., Dubey, J.P., Jenkins, M.C., Gasbarre, L., 1999. Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. Int. J. Parasitol. 29, 1647–1657.

De Meerschman, F., Focant, C., Boreux, R., Leclipteux, T., Losson, B., 2000. Cattle neosporosis in Belgium: a case-control study in dairy and beef cattle. Int. J. Parasitol. 30, 887–890.

De Meerschman, F., Speybroeck, N., Berkvens, D., Rettigner, C., Focant, C., Leclipteux, T., Cassart, D., Losson, B., 2002. Fetal infection with *Neospora caninum* in dairy and beef cattle in Belgium. Theriogenology 58, 933–945.

De Meerschman, F., Focant, C., Detry, J., Rettigner, C., Cassart, D., Losson, B., 2005. Clinical, pathological and diagnostic aspects of congenital neosporosis in a series of naturally infected calves. Vet. Rec. 157, 115–118.

Dijkstra, T., Barkema, H.W., Eysker, M., Wouda, W., 2001a. Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. Int. J. Parasitol. 31, 209–215.

Dijkstra, T., Eysker, M., Schares, G., Conraths, F.J., Wouda, W., Barkema, H.W., 2001b. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. Int. J. Parasitol. 31, 747–752.

Dijkstra, T., Barkema, H.W., Eysker, M., Beiboer, M.L., Wouda, W., 2003. Evaluation of a single serological screening of dairy herds for *Neospora caninum* antibodies. Vet. Parasitol. 110, 161–169.

Dreier, K.J., Stewarter, L.W., Kerlin, R.L., Ritter, D.M., Brake, D.A., 1999. Phenotypic characterisation of a *Neospora caninum* temperature-sensitive strain in normal and immunodeficient mice. Int. J. Parasitol. 29, 1627–1634.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Dubey, J.P., Leathers, C.W., Lindsay, D.S., 1989. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. J. Parasitol. 75, 146–148.

Dubey, J.P., Hartley, W.J., Lindsay, D.S., 1990a. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. J. Am. Vet. Med. Assoc. 197, 1043–1044.

Dubey, J.P., Miller, S., Lindsay, D.S., Topper, M.J., 1990b. *Neospora caninum*-associated myocarditis and encephalitis in an aborted calf. J. Vet. Diagn. Invest. 2, 66–69.

Dubey, J.P., Janovitz, E.B., Skowronek, A.J., 1992. Clinical neosporosis in a 4-week-old Hereford calf. Vet. Parasitol. 43, 137–141.

Dubey, J.P., De Lahunta, A., 1993. Neosporosis associated congenital limb deformities in a calf. Appl. Parasitol. 34, 229–233.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67, 1–59.

Dubey, J.P., Lindsay, D.S., Adams, D.S., Gay, J.M., Baszler, T.V., Blagburn, B.L., Thulliez, P., 1996. Serologic responses of cattle and other animals infected with *Neospora caninum*. Am. J. Vet. Res. 57, 329–336.

Dubey, J.P., Jenkins, M.C., Adams, D.S., McAllister, M.M., Anderson-Sprecher, R., Baszler, T.V., Kwok, O.C.H., Lally, N.C., Björkman, C., Uggla, A., 1997. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. J. Parasitol. 83, 1063–1069.

Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet. Parasitol. 74, 75–77.

Dubey, J.P., Abbitt, B., Topper, M.J., Edwards, J.F., 1998a. Hydrocephalus associated with *Neospora caninum*-infection in an aborted bovine fetus. J. Comp. Pathol. 118, 169–173.

Dubey, J.P., Dorough, K.R., Jenkins, M.C., Liddell, S., Speer, C.A., Kwok, O.C.H., Shen, S.K., 1998b. Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture. Int. J. Parasitol. 28, 1293–1304.

Dubey, J.P., Lindsay, D.S., 2000. Gerbils (*Meriones unguiculatus*) are highly susceptible to oral infection with *Neospora caninum* oocysts. Parasitol. Res. 86, 165–168.

Dubey, J.P., Garner, M.M., Stetter, M.D., Marsh, A.E., Barr, B.C., 2001. Acute *Sarcocystis falcatula*-like infection in a carmine bee-eater (*Merops nubicus*) and immunohistochemical cross reactivity between *Sarcocystis falcatula* and *Sarcocystis neurona*. J. Parasitol. 87, 824–832.

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkås, I., Björkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modrý, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int. J. Parasitol. 32, 929–946.

Dubey, J.P., 2003. Neosporosis in cattle. J. Parasitol. 89 (Suppl.), S42–S46.

Dubey, J.P., Sreekumar, C., Knickman, E., Miska, K.B., Vianna, M.C.B., Kwok, O.C.H., Hill, D.E., Jenkins, M.C., Lindsay, D.S., Greene, C.E., 2004. Biologic, morphologic, and molecular characterisation of *Neospora caninum* isolates from littermate dogs. Int. J. Parasitol. 34, 1157–1167.

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of bovine neosporosis. J. Comp. Pathol. 134, 267–289.

Dumètre, A., Dardé, M.L., 2003. How to detect *Toxoplasma gondii* oocysts in environmental samples? FEMS Microbiol. Rev. 27, 651–661.

Dumètre, A., Dardé, M.L., 2005. Immunomagnetic separation of *Toxoplasma gondii* oocysts using a monoclonal antibody directed against the oocyst wall. J. Microbiol. Meth. 61, 209–217.

Edelhofer, R., Löeschenberger, K., Peshke, R., Sager, H., Nowotny, N., Kolodziejek, J., Tews, A., Doneus, G., Prosl, H., 2003. First

PCR-confirmed report of a *Neospora caninum*-associated bovine abortion in Austria. Vet. Rec. 152, 471–473.

Eleni, C., Crotti, S., Manuali, E., Costarelli, S., Filippini, G., Moscati, L., Magnino, S., 2004. Detection of *Neospora caninum* in an aborted goat foetus. Vet. Parasitol. 123, 271–274.

Ellis, J.T., 1998. Polymerase chain reaction approaches for the detection of *Neospora caninum* and *Toxoplasma gondii*. Int. J. Parasitol. 28, 1053–1060.

Ellis, J.T., Amoyal, G., Ryce, C., Harper, P.A.W., Clough, K.A., Homan, W.L., Brindley, P.J., 1998. Comparison of the large subunit ribosomal DNA of *Neospora* and *Toxoplasma* and development of a new genetic marker for their differentiation based on the D2 domain. Mol. Cell. Probes 12, 1–13.

Ellis, J.T., Morrison, D.A., Liddell, S., Jenkins, M.C., Mohammed, O.B., Ryce, C., Dubey, J.P., 1999a. The genus *Hammondia* is paraphyletic. Parasitology 118, 357–362.

Ellis, J.T., McMillan, D., Ryce, C., Payne, S., Atkinson, R., Harper, P.A.W., 1999b. Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. Int. J. Parasitol. 29, 1589–1596.

Eperon, S., Brönnimann, K., Hemphill, A., Gottstein, B., 1999. Susceptibility of B-cell deficient C57BL/6 (μMT) mice to *Neospora caninum* infection. Parasite Immunol. 21, 225–236.

Esposito, M., Stettler, R., Moores, S.L., Pidathala, C., Müller, N., Stachulski, A., Berry, N.G., Rossignol, J.F., Hemphill, A., 2005. In vitro efficacies of nitazoxanide and other thiazolides against *Neospora caninum* tachyzoites reveal antiparasitic activity independent of the nitro group. Antimicrob. Agents Chemother. 49, 3715–3723.

Estill, C., 2004. In: Proceedings of the 23rd World Buiatrics Congress on Neospora-associated Abortion and Field Experience With a Commercial Vaccine in a Dairy Herd, Quebec City, Canada, July 11–16.

Ferre, I., Aduriz, G., del-Pozo, I., Regidor-Cerrillo, J., Atxaerandio, R., Collantes-Fernández, E., Hurtado, A., Ugarte-Garagalza, C., Ortega-Mora, L.M., 2005. Detection of *Neospora caninum* in the semen and blood of naturally infected bulls. Theriogenology 63, 1504–1518.

Fioretti, D.P., Rosignoli, L., Ricci, G., Moretti, A., Pasquali, P., Polidori, G.A., 2000. *Neospora caninum* infection in a clinically healthy calf: parasitological study and serological follow-up. J. Vet. Med. B 47, 47–53.

Fioretti, D.P., Pasquai, P., Diaferia, M., Mangili, V., Rosignoli, L., 2003. *Neospora caninum* infection and congenital transmission: serological and parasitological study of cows up to the fourth gestation. J. Vet. Med. B 50, 399–404.

Frössling, J., Bonnett, B., Lindberg, A., Björkman, C., 2003. Validation of a *Neospora caninum* iscom ELISA without a gold standard. Prev. Vet. Med. 57, 141–153.

Frössling, J., Lindberg, A., Björkman, C., 2006. Evaluation of an iscom ELISA used for detection of antibodies to Neospora caninum in bulk milk. Prev. Vet. Med. 74, 120–129.

Gaturaga, I., Chahan, B., Xuan, X., Huang, X., Liao, M., Fukumoto, S., Hirata, H., Nishikawa, Y., Takashima, Y., Suzuki, H., Fujisaki, K., Sugimoto, C., 2005. Detection of antibodies to *Neospora caninum* in cattle by enzyme-linked immunosorbent assay with truncated NcSR2 expressed in *Escherichia coli*. J. Parasitol. 91, 191–192.

Gondim, L.F.P., Saeki, H., Onaga, H., Haritani, M., Yamane, I., 1999. Maintenance of *Neospora caninum* tachyzoites using Mongolian gerbils (*Meriones unguiculatus*). N.Z. Vet. J. 47, 36.

Gondim, L.F.P., Pinheiro, A.M., Santos, P.O.M., Jesus, E.E.V., Ribeiro, M.B., Fernandes, H.S., Almeida, M.A.O., Freire, S.M., Meyer, R., McAllister, M.M., 2001. Isolation of *Neospora caninum* from the brain of a naturally infected dog, and production of encysted bradyzoites in gerbils. Vet. Parasitol. 101, 1–7.

Gondim, L.F.P., Gao, L., McAllister, M.M., 2002. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. J. Parasitol. 88, 1159–1163.

Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004a. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.

Gondim, L.F.P., McAllister, M.M., Anderson-Sprecher, R.C., Björkman, C., Lock, T.F., Firkins, L.D., Gao, L., Fischer, W.R., 2004b. Transplacental transmission and abortion in cows administered *Neospora caninum* oocysts. J. Parasitol. 90, 1394–1400.

Gottstein, B., Hentrich, B., Wyss, R., Thür, B., Busato, A., Stärk, K.D.C., Müller, N., 1998. Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. Int. J. Parasitol. 28, 679–691.

Gottstein, B., Hentrich, B., Wyss, R., Thür, B., Bruckner, L., Müller, N., Kaufmann, H., Waldvogel, A., 1999. Molekular- und immundiagnostische Untersuchungen zur bovinen Neosporose in der Schweiz. Schweiz. Arch. Tierheilkd. 141, 59–68.

Gottstein, B., Eperon, S., Dai, W.J., Cannas, A., Hemphill, A., Greif, G., 2001. Efficacy of toltrazuril and ponazuril against experimental *Neospora caninum* infection in mice. Parasitol. Res. 87, 43–48.

Greiner, M., Gardner, I.A., 2000. Application of diagnostic tests in veterinary epidemiologic studies. Prev. Vet. Med. 45, 43–59.

Guy, C.S., Williams, D.J.L., Kelly, D.F., McGarry, J.W., Guy, F., Björkman, C., Smith, R.F., Trees, A.J., 2001. *Neospora caninum* in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody. Vet. Rec. 149, 443–449.

Hall, C.A., Reichel, M.P., Ellis, J.T., 2005. *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. Vet. Parasitol. 128, 231–241.

Harkins, D., Clements, D.N., Maley, S., Marks, J., Wright, S., Esteban, I., Innes, E.A., Buxton, D., 1998. Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii*. J. Comp. Pathol. 119, 45–55.

Hattel, A.L., Castro, M.D., Gummo, J.D., Weinstock, D., Reed, J.A., Dubey, J.P., 1998. Neosporosis-associated bovine abortion in Pennsylvania. Vet. Parasitol. 74, 307–313.

Hässig, M., Gottstein, B., 2002. Epidemiological investigations of abortions due to *Neospora caninum* on Swiss dairy farms. Vet. Rec. 150, 538–542.

Hässig, M., Sager, H., Reitt, K., Ziegler, D., Strabel, D., Gottstein, B., 2003. *Neospora caninum* i sheep: a herd case report. Vet. Parasitol. 117, 213–220.

Helman, R.G., Stair, E.L., Lehenbauer, T.W., Rodgers, S., Saliki, J.T., 1998. Neosporal abortion in Oklahoma cattle with emphasis on the distribution of brain lesions in aborted fetuses. J. Vet. Diagn. Invest. 10, 292–295.

Henning, K., Schares, G., Granzow, H., Polster, U., Hartmann, M., Hotzel, H., Sachse, K., Peters, M., Rauser, M., 2002. *Neospora caninum* and *Waddlia chondrophila* strain 2032/99 in a septic stillborn calf. Vet. Microbiol. 85, 285–292.

Hernandez, J., Risco, C., Donovan, A., 2001. Association between exposure to *Neospora caninum* and milk production in dairy cows. J. Am. Vet. Med. Assoc. 219, 632–635.

Hernandez, J., Risco, C., Donovan, A., 2002. Risk of abortion associated with *Neospora caninum* during different lactations and evidence of congenital transmission in dairy cows. J. Am. Vet. Med. Assoc. 221, 1742–1746.

Heuer, C., Nicholson, C.R., Muñoz Bielsa, J., Weston, J.F., 2003. In: Proceedings of the 10th International Symposium for Veterinary Epidemiology and Economics on Efficacy of a Vaccine Against *Neospora caninum* Related Abortions in New Zealand Dairy Herds, Vina del Mar, Chile, November 17–21.

Hietala, S.K., Thurmond, M.C., 1999. Postnatal *Neospora caninum* transmission and transient serologic responses in two dairies. Int. J. Parasitol. 29, 1669–1676.

Hill, D.E., Liddell, S., Jenkins, M.C., Dubey, J.P., 2001. Specific detection of *Neospora caninum* oocyst in fecal samples from experimentally-infected dogs using the polymerase chain reaction. J. Parasitol. 87, 395–398.

Ho, M.S.Y., Barr, B.C., Marsh, A.E., Anderson, M.L., Rowe, J.D., Tarantal, A.F., Hendrickx, A.G., Sverlow, K., Dubey, J.P., Conrad, P.A., 1996. Identification of bovine *Neospora* parasites by PCR amplification and specific small subunit rRNA sequence probe hybridization. J. Clin. Microbiol. 34, 1203–1208.

Ho, M.S.Y., Barr, B.C., Rowe, J.D., Anderson, M.L., Sverlow, K.W., Packham, A., Marsh, A.E., Conrad, P.A., 1997a. Detection of *Neospora* sp. from infected bovine tissues by PCR and probe hybridization. J. Parasitol. 83, 508–514.

Ho, M.S.Y., Barr, B.C., Tarantal, A.F., Lai, L.T.Y., Hendrickx, A.G., Marsh, A.E., Sverlow, K.W., Packham, A.E., Conrad, P.A., 1997b. Detection of *Neospora* from tissues of experimentally infected rhesus macaques by PCR and specific DNA probe hybridization. J. Clin. Microbiol. 35, 1740–1745.

Hobson, J.C., Duffield, T.F., Kelton, D., Lissemore, K., Hietala, S.K., Leslie, K.E., McEwen, B., Cramer, G., Peregrine, A.S., 2002. *Neospora caninum* serostatus and milk production of Holstein cattle. J. Am. Vet. Med. Assoc. 221, 1160–1164.

Hobson, J.C., Duffield, T.F., Kelton, D., Lissemore, K., Hietala, S.K., Leslie, K.E., McEwen, B., Peregrine, A.S., 2005. Risk factors associated with *Neospora caninum* abortion in Ontario Holstein dairy herds. Vet. Parasitol. 127, 177–188.

Holmdahl, O.J.M., Mattsson, J.G., 1996. Rapid and sensitive identification of *Neospora caninum* by in vitro amplification of the internal transcribed spacer 1. Parasitology 112, 177–182.

Hoorfar, J., Wolffs, P., Radstrom, P., 2004. Diagnostic PCR: validation and sample preparation are two sides of the same coin. Acta Pathol. Microbiol. Immunol. Scand. 112, 808–814.

Howe, D.K., Crawford, A.C., Lindsay, D., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infect. Immun. 66, 5322–5328.

Howe, D.K., Tang, K., Conrad, P.A., Sverlow, K., Dubey, J.P., Sibley, L.D., 2002. Sensitive and specific identification of *Neospora caninum* infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clin. Diagn. Lab. Immunol. 9, 611–615.

Huang, C.C., Yang, C.H., Watanabe, Y., Liao, Y.K., Ooi, H.K., 2004a. Finding of *Neospora caninum* in the wild brown rat (*Rattus norvegicus*). Vet. Res. 35, 283–290.

Huang, C.C., Ting, L.J., Shiau, J.R., Chen, M.C., Ooi, H.K., 2004b. An abortion storm in cattle associated with neosporosis in Taiwan. J. Vet. Med. Sci. 66, 465–467.

Hughes, J.M., Williams, R.H., Morley, E.K., Cook, D.A., Terry, R.S., Murphy, R.G., Smith, J.E., Hide, G., 2006. The prevalence of *Neospora caninum* and co-infection with *Toxoplasma gondii* by PCR analysis in naturally occurring mammal populations. Parasitology 132, 29–36.

Hůrková, L., Halova, D., Modrý, D., 2005. The prevalence of *Neospora caninum* antibodies in bulk milk of dairy herds in the Czech Republic: a case report. Vet. Med. (Czech) 50, 549–552.

Hůrková, L., Modrý, D., 2006. PCR detection of *Neospora caninum*, *Toxoplasma gondii* and *Encephalitozoon cuniculi* in brains of wild carnivores. Vet. Parasitol. 137, 150–154.

Innes, E.A., Wright, S.E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I.M., Buxton, D., 2001. Protection against vertical transmission in bovine neosporosis. Int. J. Parasitol. 31, 1523–1534.

Innes, E.A., Wright, S., Bartley, P., Maley, S., Macaldowie, C., Esteban-Redondo, I., Buxton, D., 2005. The host–parasite relationship in bovine neosporosis. Vet. Immunol. Immunopathol. 108, 29–36.

Jakubek, E.B., Uggla, A., 2005. Persistence of *Neospora caninum*-specific immunoglobulin G antibodies in bovine blood and lung tissue stored at room temperature. J. Vet. Diagn. Invest. 17, 458–460.

Jenkins, M.C., Wouda, W., Dubey, J.P., 1997. Serological response over time to recombinant *Neospora caninum* antigens in cattle after a neosporosis-induced abortion. Clin. Diagn. Lab. Immunol. 4, 270–274.

Jenkins, M.C., Caver, J.A., Björkman, C., Anderson, T.C., Romand, S., Vinyard, B., Uggla, A., Thulliez, P., Dubey, J.P., 2000. Serological investigation of an outbreak of *Neospora caninum*-associated abortion in a dairy herd in southeastern United States. Vet. Parasitol. 94, 17–26.

Jenkins, M., Baszler, T., Björkman, C., Schares, G., Williams, D., 2002. Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int. J. Parasitol. 32, 631–636.

Jenkins, M.C., Fetterer, R., Schares, G., Björkman, C., Wapenaar, W., McAllister, M., Dubey, J.P., 2005. HPLC purification of recombinant NCGRA6 antigen improves enzyme-linked immunosorbent assay for serodiagnosis of bovine neosporosis. Vet. Parasitol. 131, 227–234.

Kaufmann, H., Yamage, M., Roditi, I., Dobbelaere, D., Dubey, J.P., Holmdahl, O.J.M., Trees, A., Gottstein, B., 1996. Discrimination of *Neospora caninum* from *Toxoplasma gondii* and other

apicomplexan parasites by hybridization and PCR. Mol. Cell. Probes 10, 289–297.

Kim, J.H., Sohn, H.J., Hwang, E.K., Hwang, W.S., Hur, K., Jean, Y.H., Lee, B.C., Rhee, J.C., Kang, Y.B., Yamane, I., Kim, D.J., 1998a. In vitro isolation of a bovine *Neospora* in Korea. Korean J. Vet. Res. 38, 139–145.

Kim, J.H., Hwang, E.K., Sohn, H.J., Jean, Y.H., Yoon, S.S., Kim, D.Y., 1998b. Repeated bovine abortion associated with *Neospora caninum* in Korea. Korean J. Vet. Res. 38, 853–858.

Kim, J.H., Sohn, H.J., Hwang, W.S., Hwang, E.K., Jean, Y.H., Yamane, I., Kim, D.Y., 2000. In vitro isolation and characterization of bovine *Neospora caninum* in Korea. Vet. Parasitol. 90, 147–154.

Kim, J.H., Lee, J.K., Lee, B.C., Park, B.K., Yoo, H.S., Hwang, W.S., Shin, N.R., Kang, M.S., Jean, Y.H., Yoon, H.J., Kang, S.K., Kim, D.Y., 2002. Diagnostic survey of bovine abortion in Korea: with special emphasis on *Neospora caninum*. J. Vet. Med. Sci. 64, 1123–1127.

Kobayashi, Y., Yamada, M., Omata, Y., Koyama, T., Saito, A., Matsuda, T., Okuyama, K., Fujimoto, S., Furuoka, H., Matsui, T., 2001. Naturally-occurring *Neospora caninum* infection in an adult sheep and her twin fetuses. J. Parasitol. 87, 434–436.

Koiwai, M., Hamaoka, T., Haritani, M., Shimizu, S., Kimura, K., Yamane, I., 2005. Proportion of abortions due to neosporosis among dairy cattle in Japan. J. Vet. Med. Sci. 67, 1173–1175.

Koyama, T., Kobayashi, Y., Omata, Y., Yamada, M., Furuoka, H., Maeda, R., Matsui, T., Saito, A., Mikami, T., 2001. Isolation of *Neospora caninum* from the brain of a pregnant sheep. J. Parasitol. 87, 1486–1488.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996a. Development of a polymerase chain reaction assay for the diagnosis of neosporosis using the *Neospora caninum* 14–3–3 gene. Mol. Biochem. Parasitol. 75, 169–178.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996b. Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. Clin. Diagn. Lab. Immunol. 3, 275–279.

Landmann, J.K., Jillella, D., O'Donoghue, P.J., McGowan, M.R., 2002. Confirmation of the prevention of vertical transmission of *Neospora caninum* in cattle by the use of embryo transfer. Aust. Vet. J. 80, 502–503.

Lemberger, K.Y., Gondim, L.F.P., Pessier, A.P., McAllister, M.M., Kinsel, M.J., 2005. *Neospora caninum* infection in a free-ranging raccoon (*Procyon lotor*) with concurrent canine distemper virus infection. J. Parasitol. 91, 960–961.

Liao, M., Zhang, S., Xuan, S., Zhang, G., Huang, X., Igarashi, I., Fujisaki, K., 2005. Development of rapid immunochromatographic test with recombinant NcSAG1 for detection of antibodies to *Neospora caninum* in cattle. Clin. Diagn. Lab. Immunol. 12, 885–887.

Lei, Y., Davey, M., Ellis, J.T., 2005. Attachment and invasion of *Toxoplasma gondii* and *Neospora caninum* to epithelial and fibroblast cell lines in vitro. Parasitology 131, 583–590.

Liddell, S., Jenkins, M.C., Collica, C.M., Dubey, J.P., 1999a. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. J. Parasitol. 85, 1072–1075.

Liddell, S., Jenkins, M.C., Dubey, J.P., 1999b. A competitive PCR assay for quantitative detection of *Neospora caninum*. Int. J. Parasitol. 29, 1583–1587.

Liddell, S., Jenkins, M.C., Dubey, J.P., 1999c. Vertical transmission of *Neospora caninum* in BALB/c mice determined by polymerase chain reaction detection. J. Parasitol. 85, 550–555.

Lindsay, D.S., Dubey, J.P., 1989a. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am. J. Vet. Res. 50, 1981–1983.

Lindsay, D.S., Dubey, J.P., 1989b. *Neospora caninum* (Protozoa: Apicomplexa) infections in mice. J. Parasitol. 75, 772–779.

Lindsay, D.S., Dubey, J.P., 1989c. In vitro development of *Neospora caninum* (Protozoa: Apicomplexa) from dogs. J. Parasitol. 75, 163–165.

Lindsay, D.S., Upton, S.J., Dubey, J.P., 1999a. A structural study of the *Neospora caninum* oocyst. Int. J. Parasitol. 29, 1521–1523.

Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999b. Confirmation that the dog is a definitive host for *Neospora caninum*. Vet. Parasitol. 82, 327–333.

Lindsay, D.S., Ritter, D.M., Brake, D., 2001. Oocyst excretion in dogs fed mouse brains containing tissue cysts of a cloned line of *Neospora caninum*. J. Parasitol. 87, 909–911.

Lindsay, D.S., Lenz, S.D., Cole, R.A., Dubey, J.P., Blagburn, B.L., 1995. Mouse model for central nervous system *Neospora caninum* infections. J. Parasitol. 81, 313–315.

Locatelli-Dittrich, R., Richartz, R.R.T.B., Gasino-Joineau, M.E., Pinckney, R.D., de Sousa, R.S., Leite, L.C., Thomaz-Soccol, V., 2003. Isolation of *Neospora caninum* from a blind calf in Paraná, southern Brazil. Vet. Rec. 153, 366–367.

Locatelli-Dittrich, R., Thomaz-Soccol, V., Richartz, R.R.T.B., Gasino-Joineau, M.E., van der Vinne, R., Pinckney, R.D., 2004. Isolamento de *Neospora caninum* de feto bovino de rebanho leiteiro no Paraná. Revista Brasil. Parasitol. Vet. 13, 103–109.

Long, M.T., Baszler, T.V., 1996. Fetal loss in Balb/c mice infected with *Neospora caninum*. J. Parasitol. 82, 608–611.

Long, M.T., Baszler, T.V., Mathison, B.A., 1998. Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*. J. Parasitol. 84, 316–320.

Louie, K., Sverlow, K.W., Barr, B.C., Anderson, M.L., Conrad, P.A., 1997. Cloning and characterization of two recombinant *Neospora* protein fragments and their use in serodiagnosis of bovine neosporosis. Clin. Diagn. Lab. Immunol. 4, 692–699.

Löschenberger, K., Szölgyényi, W., Peschke, R., Prosl, H., 2004. Detection of the protozoan *Neospora caninum* using in situ polymerase chain reaction. Biotech. Histochem. 79, 101–105.

Magnino, S., Vigo, P.G., Fabbi, M., Colombo, M., Bandi, C., Genchi, C., 1999. Isolation of a bovine *Neospora* from a newborn calf in Italy. Vet. Rec. 144, 456.

Magnino, S., Vigo, P.G., Bandi, C., Bazzocchi, C., Fabbi, M., Genchi, C., 2000. Small-subunit rDNA sequencing of the Italian bovine *Neospora caninum* isolate (NC-PV1 strain). Parassitologia 42, 191–192.

Mainar-Jaime, R.C., Thurmond, M.C., Berzal-Herranz, B., Hietala, S.K., 1999. Seroprevalence of *Neospora caninum* and abortion in dairy cows in northern Spain. Vet. Rec. 145, 72–75.

Maley, S.W., Buxton, D., Thomson, K.M., Schriefer, C.E.S., Innes, E.A., 2001. Serological analysis of calves experimentally infected with *Neospora caninum*: a 1-year study. Vet. Parasitol. 96, 1–9.

Marsh, A.E., Barr, B.C., Sverlow, K., Ho, M., Dubey, J.P., Conrad, P.A., 1995. Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. J. Parasitol. 81, 530–535.

Marsh, A.E., Barr, B.C., Packham, A.E., Conrad, P.A., 1998. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). J. Parasitol. 84, 983–991.

McAllister, M., Huffman, E.M., Hietala, S.K., Conrad, P.A., Anderson, M.L., Salman, M.D., 1996a. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. J. Vet. Diagn. Invest. 8, 355–357.

McAllister, M.M., Parmley, S.F., Weiss, L.M., Welch, V.J., McGuire, A.M., 1996b. An immunohistochemical method for detecting bradyzoite antigen (BAG5) in *Toxoplasma gondii*-infected tissues cross-reacts with a *Neospora caninum* bradyzoite antigen. J. Parasitol. 82, 354–355.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.

McAllister, M., Wills, R.A., McGuire, A.M., Jolley, W.R., Tranas, J.D., Williams, E.S., Lindsay, D.S., Björkman, C., Belden, E.L., 1999. Ingestion of *Neospora caninum* tissue cysts by *Mustela* species. Int. J. Parasitol. 29, 1531–1536.

McAllister, M.M., Björkman, C., Anderson-Sprecher, R., Rogers, D.G., 2000. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. J. Am. Vet. Med. Assoc. 217, 881–887.

McAllister, M.M., Wallace, R.L., Björkman, C., Gao, L., Firkins, L.D., 2005. A probable source of *Neospora caninum* infection in an abortion outbreak in dairy cows. Bovine Pract. 39, 69–74.

McGarry, J.W., Guy, F., Trees, A.J., Williams, D.J.L., Davison, H.C., Björkman, C., 2000. Validation and application of an inhibition ELISA to detect serum antibodies to *Neospora caninum* in different host species. Int. J. Parasitol. 30, 880–884.

McGarry, J.W., Stockton, C.M., Williams, D.J.L., Trees, A.J., 2003. Protracted shedding of oocysts of *Neospora caninum* by a naturally infected foxhound. J. Parasitol. 89, 628–630.

McGuire, A.M., McAllister, M.M., Jolley, W.R., 1997. Separation and cryopreservation of *Neospora caninum* tissue cysts from murine brain. J. Parasitol. 83, 319–321.

McGuire, A.M., McAllister, M., Wills, R.A., Tranas, J.D., 1999. Experimental inoculation of domestic pigeons (*Columbia livia*) and zebra finches (*Poephila guttata*) with *Neospora caninum* tachyzoites. Int. J. Parasitol. 29, 1525–1529.

Medina, L., Cruz-Vazquez, C., Quezada, T., Morales, E., Garcia-Vazquez, Z., 2006. Survey of *Neospora caninum* infection by nested PCR in aborted fetuses from dairy farms in Aguascalientes, Mexico. Vet. Parasitol. 136, 187–191.

Meseck, E.K., Njaa, B.L., Haley, N.J., Park, E.H., Barr, S.C., 2005. Use of a multiplex polymerase chain reaction to rapidly differentiate *Neospora caninum* from *Toxoplasma gondii* in an adult dog with necrotizing myocarditis and myocardial infarct. J. Vet. Diagn. Invest. 17, 565–568.

Miller, C.M.D., Quinn, H.E., Windsor, P.A., Ellis, J.T., 2002. Characterization of the first Australian isolate of *Neospora caninum* form cattle. Aust. Vet. J. 80, 620–625.

Moen, A.R., Wouda, W., Mul, M.F., Graat, E.A.M., van Werven, T., 1998. Increased risk of abortion following *Neospora caninum* abortion outbreaks: a retrospective and prospective cohort study in four dairy herds. Theriogenology 49, 1301–1309.

Moore, D.P., Campero, C.M., Odeon, A.C., Chayer, R., Bianco, M.A., 2003. Reproductive losses due to *Neospora caninum* in a beef herd in Argentina. J. Vet. Med. B: Infect. Dis. Vet. Publ. Health 50, 304–308.

Moore, D.P., Leunda, M.R., Zamorano, P.I., Odeón, A.C., Romera, S.A., Cano, A., de Yaniz, G., Venturini, M.C., Campero, C.M., 2005. Immune response to *Neospora caninum* in naturally infected heifers and heifers vaccinated with inactivated antigen during the second trimester of gestation. Vet. Parasitol. 130, 29–39.

Moskwa, B., Cabaj, W., Pastusiak, K., Bien, J., 2003. The suitability of milk in detection of *Neospora caninum* infection in cows. Acta Parasitol. 48, 138–141.

Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J. Clin. Microbiol. 34, 2850–2852.

Müller, N., Sager, H., Hemphill, A., Mehlhorn, H., Heydorn, A.O., Gottstein, B., 2001. Comparative molecular investigation of Nc5-PCR amplicons from *Neospora caninum* NC-1 and *Hammondia heydorni*-Berlin-1996. Parasitol. Res. 87, 883–885.

Müller, N., Vonlaufen, N., Gianinazzi, C., Leib, S.L., Hemphill, A., 2002. Application of real-time fluorescent PCR for quantitative assessment of *Neospora caninum* infections in organotypic slice cultures of rat central nervous system tissue. J. Clin. Microbiol. 40, 252–255.

Naguleswaran, A., Müller, N., Hemphill, A., 2003. *Neospora caninum* and *Toxoplasma gondii*: a novel adhesion/invasion assay reveals distinct differences in tachyzoite–host cell interactions. Exp. Parasitol. 104, 149–158.

Nietfeld, J.C., Dubey, J.P., Anderson, M.L., Libal, M.C., Yaeger, M.J., Neiger, R.D., 1992. *Neospora*-like protozoan infection as a cause of abortion in dairy cattle. J. Vet. Diagn. Invest. 4, 223–226.

Nishikawa, Y., Kousaka, Y., Tragoolpua, K., Xuan, X., Makala, L., Fujisaki, K., Mikami, T., Nagasawa, H., 2001. Characterization of *Neospora caninum* surface protein NcSRS2 based on baculovirus expression system and its application for serodiagnosis of Neospora infection. J. Clin. Microbiol. 39, 3987–3991.

Okeoma, C.M., Williamson, N.B., Pomroy, W.E., Stowell, K.M., Gillespie, L., 2004a. The use of PCR to detect *Neospora caninum* DNA in the blood of naturally infected cows. Vet. Parasitol. 122, 307–315.

Okeoma, C.M., Williamson, N.B., Pomroy, W.E., Stowell, K.M., Gillespie, L.M., 2004b. Isolation and molecular characterisation of *Neospora caninum* in cattle in New Zealand. N.Z. Vet. J. 52, 364–370.

Ortega-Mora, L.M., Ferre, I., del Pozo, I., Caetano da Silva, A., Collantes-Fernández, E., Regidor-Cerrillo, J., Ugarte-Garagalza, C., Aduriz, G., 2003. Detection of *Neospora caninum* in semen of bulls. Vet. Parasitol. 117, 301–308.

O'Toole, D., Jeffrey, M., 1987. Congenital sporozoan encephalomyelitis in a calf. Vet. Rec. 121, 563–566.

Osawa, T., Wastling, J., Maley, S., Buxton, D., Innes, E.A., 1998. A multiple antigen ELISA to detect *Neospora*-specific antibodies in bovine sera, bovine foetal fluids, ovine and caprine sera. Vet. Parasitol. 79, 19–34.

Otter, A., Jeffrey, M., Griffiths, I.B., Dubey, J.P., 1995. A survey of the incidence of *Neospora caninum* infection in aborted and stillborn bovine fetuses in England and Wales. Vet. Rec. 136, 602–606.

Otter, A., Jeffrey, M., Scholes, S.F.E., Helmick, B., Wilesmith, J.W., Trees, A.J., 1997. Comparison of histology with maternal and fetal serology for the diagnosis of abortion due to bovine neosporosis. Vet. Rec. 141, 487–489.

Ould-Amrouche, A., Klein, F., Osdoit, C., Mohamed, H.O., Touratier, A., Sanaa, M., Mialot, J.P., 1999. Estimation of *Neospora caninum* seroprevalence in dairy cattle from Normandy, France. Vet. Res. 30, 531–538.

Packham, A.E., Sverlow, K.W., Conrad, P.A., Loomis, E.F., Rowe, J.D., Anderson, M.L., Marsh, A.E., Cray, C., Barr, B.C., 1998. A modified agglutination test for *Neospora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 5, 467–473.

Paré, J., Hietala, S.K., Thurmond, M.C., 1995. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J. Vet. Diagn. Invest. 7, 352–359.

Paré, J., Thurmond, M.C., Hietala, S.K., 1996. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can. J. Vet. Res. 60, 133–139.

Paré, J., Thurmond, M.C., Hietala, S.K., 1997. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. J. Parasitol. 83, 82–87.

Paré, J., Fecteau, G., Fortin, M., Marsolais, G., 1998. Seroepidemiologic study of *Neospora caninum* in dairy herds. J. Am. Vet. Med. Assoc. 213, 1595–1598.

Parish, S.M., Maag-Miller, L., Besser, T.E., Weidner, J.P., McElwain, T., Knowles, D.P., Leathers, C.W., 1987. Myelitis associated with protozoal infection in newborn calves. J. Am. Vet. Med. Assoc. 191, 1599–1600.

Payne, S., Ellis, J., 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. Int. J. Parasitol. 26, 347–351.

Pereira-Bueno, J., Quintanilla-Gozalo, A., Seijas-Carballedo, A., Costas, E., Ortega-Mora, L.M., 2000. Observational studies in *Neospora caninum* infected dairy cattle: pattern of transmission and age-related antibody fluctuations. Int. J. Parasitol. 30, 906–909.

Pereira-Bueno, J., Quintanilla-Gozalo, A., Perez-Perez, V., Espi-Felgueroso, A., Álvarez, G., Collantes-Fernández, E., Ortega-Mora, L.M., 2003. Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. Vet. Parasitol. 111, 143–152.

Peters, M., Wagner, F., Schares, G., 2000. Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. Parasitol. Res. 86, 1–7.

Peters, M., Lütkefels, E., Heckeroth, A.R., Schares, G., 2001a. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. Int. J. Parasitol. 31, 1144–1148.

Peters, M., Wohlsein, P., Knieriem, A., Schares, G., 2001b. *Neospora caninum* infection associated with stillbirths in captive antelopes (*Tragelaphus imberbis*). Vet. Parasitol. 97, 153–157.

Pfeiffer, D.U., Williamson, N.B., Reichel, M.P., Wichtel, J.J., Teague, W.R., 2002. A longitudinal study of *Neospora caninum* infection on a dairy farm in New Zealand. Prev. Vet. Med. 54, 11–24.

Pipano, E., Shkap, V., Fish, L., Savitsky, I., Perl, S., Orgad, U., 2002. Susceptibility of *Psammomys obesus* and *Meriones tristrami* to tachyzoites of *Neospora caninum*. J. Parasitol. 88, 314–319.

Pitel, P.H., Pronost, S., Gargala, G., Anrioud, D., Toquet, M.-P., Foucher, N., Collobert-Laugier, C., Fortier, G., Ballet, J.-J., 2002. Detection of *Sarcocystis neurona* antibodies in French horses with neurological signs. Int. J. Parasitol. 32, 481–485.

Quintanilla-Gozalo, A., Pereira-Bueno, J., Seijas-Carballedo, A., Costas, E., Ortega-Mora, L.M., 2000. Observational studies in *Neospora caninum* infected dairy cattle: relationship infection–abortion and gestational antibody fluctuations. Int. J. Parasitol. 30, 900–906.

Ramamoorthy, S., Sriranganathan, N., Lindsay, D.S., 2005. Gerbil model of acute neosporosis. Vet. Parasitol. 127, 111–114.

Reichel, M.P., Drake, J.M., 1996. The diagnosis of *Neospora* abortions in cattle. N.Z. Vet. J. 44, 151–154.

Reichel, M.P., Pfeiffer, D.U., 2002. An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. Vet. Parasitol. 107, 197–207.

Reichel, M.P., Thornton, R.N., Morgan, P.L., Mills, R.J.M., Schares, G., 1998. Neosporosis in a pup. N.Z. Vet. J. 46, 106–110.

Rettigner, C., De Meerschman, F., Focant, C., Vanderplasschen, A., Losson, B., 2004. The vertical transmission following the reactivation of a *Neospora caninum* chronic infection does not seem to be due an alteration of the systemic immune response in pregnant CBA/Ca mice. Parasitology 128, 149–160.

Rodrigues, A.A.R., Gennari, S.M., Aguiar, D.M., Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C.B., Dubey, J.P., 2004. Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Vet. Parasitol. 124, 139–150.

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.

Romero, J.J., Pérez, E., Frankena, K., 2004. Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions. Vet. Parasitol. 123, 149–159.

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audigé, L., Gottstein, B., 2001. A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. Vet. Parasitol. 102, 1–15.

Sager, H., Gloor, M., Björkman, C., Kritzner, S., Gottstein, B., 2003. Assessment of antibody avidity in aborting cattle by a somatic *Neospora caninum* tachyzoite antigen IgG avidity ELISA. Vet. Parasitol. 112, 1–10.

Sager, H., Hüssy, D., Kuffer, A., Schreve, F., Gottstein, B., 2005. Mise en évidence d'un de ''abortion strom'' (transmission transplacentaire exogéne de *Neospora caninum*) dans une exploitation de vaches laitières: une première en Suisse. Schweiz. Arch. Tierheilkd. 147, 113–120.

Sawada, M., Kondo, H., Tomioka, Y., Park, C.H., Morita, T., Shimada, A., Umemura, T., 2000. Isolation of *Neospora caninum* from the brain of a naturally infected adult dairy cow. Vet. Parasitol. 90, 247–252.

Schares, G., Peters, M., Wurm, R., Tackmann, K., Henning, K., Conraths, F.J., 1997. *Neospora caninum* verursacht Aborte in einem Rinderbestand in Nordrhein-Westfalen. Dtsch. Tierarztl. Wochenschr. 104, 208–212.

Schares, G., Peters, M., Wurm, R., Bärwald, A., Conraths, F.J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87–98.

Schares, G., Conraths, F.J., Reichel, M.P., 1999a. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. Int. J. Parasitol. 29, 1659–1667.

Schares, G., Dubremetz, J.F., Dubey, J.P., Bärwald, A., Loyens, A., Conraths, F.J., 1999b. *Neospora caninum*: identification of 19-, 38-, and 40-kDa surface antigens and a 33-kDa dense granule antigen using monoclonal antibodies. Exp. Parasitol. 92, 109–119.

Schares, G., Rauser, M., Zimmer, K., Peters, M., Wurm, R., Dubey, J.P., de Graaf, D.C., Edelhofer, R., Mertens, C., Hess, G., Conraths, F.J., 1999c. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J. Parasitol. 85, 688–694.

Schares, G., Rauser, M., Söndgen, P., Rehberg, P., Bärwald, A., Dubey, J.P., Edelhofer, R., Conraths, F.J., 2000. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. Int. J. Parasitol. 30, 1123–1130.

Schares, G., Bärwald, A., Staubach, C., Söndgen, P., Rauser, M., Schröder, R., Peters, M., Wurm, R., Selhorst, T., Conraths, F.J., 2002. p38-avidity-ELISA: examination of herds experiencing epidemic or endemic *Neospora caninum*-associated bovine abortion. Vet. Parasitol. 106, 293–305.

Schares, G., Bärwald, A., Staubach, C., Ziller, M., Klöss, D., Wurm, R., Rauser, M., Labohm, R., Dräger, K., Fasen, W., Hess, R.G., Conraths, F.J., 2003. Regional distribution of bovine *Neospora caninum* infection in the German state of Rhineland-Palatinate modelled by logistic regression. Int. J. Parasitol. 33, 1631–1640.

Schares, G., Bärwald, A., Staubach, C., Ziller, M., Klöss, D., Schroder, R., Labohm, R., Dräger, K., Fasen, W., Hess, R.G., Conraths, F.J., 2004a. Potential risk factors for bovine *Neospora caninum* infection in Germany are not under the control of the farmers. Parasitology 129, 301–309.

Schares, G., Bärwald, A., Staubach, C., Wurm, R., Rauser, M., Conraths, F.J., Schroeder, C., 2004b. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. Vet. Parasitol. 120, 55–63.

Schares, G., Bärwald, A., Conraths, F.J., 2005a. Adaptation of a surface antigen-based ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. J. Vet. Med. B 52, 45–48.

Schares, G., Pantchev, N., Barutzki, D., Heyddorn, A.O., Bauer, C., Conraths, F., 2005b. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasit. 1525–1537.

Schatzberg, S.J., Haley, N.J., Barr, S.C., De Lahunta, A., Olby, N., Munana, K., Sharp, N.J.H., 2003. Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. Am. J. Vet. Res. 64, 1507–1513.

Serrano, E., Ferre, I., Osoro, K., Aduriz, G., Mateos-Sanz, A., Martinez, A., Atxaerandio, R., Hidalgo, C.O., Ortega-Mora, L.M., 2006. Intrauterine *Neospora caninum* inoculation of heifers. Vet. Parasitol. 135, 197–203.

Sharma, S.P., Dubey, J.P., 1981. Quantitative survival of *Toxoplasma gondii* tachyzoites and bradyzoites in pepsin and in trypsin solutions. Am. J. Vet. Res. 42, 128–130.

Shivaprasad, H.L., Ely, R., Dubey, J.P., 1989. A *Neospora*-like protozoon found in an aborted bovine placenta. Vet. Parasitol. 34, 145–148.

Schock, A., Buxton, D., Spence, J.A., Baird, A., Low, J.C., 2000. Histopathological survey of aborted bovine fetuses in Scotland with special reference to *Neospora caninum*. Vet. Rec. 147, 687–688.

Šlapeta, J.R., Koudela, B., Votypka, J., Modry, D., Horejs, R., Lukes, J., 2002a. Coprodiagnosis of *Hammondia heydorni* in dogs by PCR based amplification of ITS 1 rRNA: differentiation from morphologically indistinguishable oocysts of *Neospora caninum*. Vet. J. 163, 147–154.

Šlapeta, J.R., Modry, D., Kyselova, I., Horejs, R., Lukes, J., Koudela, B., 2002b. Dog shedding oocysts of *Neospora caninum*: PCR diagnosis and molecular phylogenetic approach. Vet. Parasitol. 109, 157–167.

Slotved, H.C., Jensen, L., Lind, P., 1999. Comparison of the IFAT and Iscom-ELISA response in bovine foetuses with *Neospora caninum* infection. Int. J. Parasitol. 29, 1165–1174.

Spencer, J.A., Witherow, A.K., Blagburn, B.L., 2000. A random amplified polymorphic DNA polymerase chain reaction technique that differentiates between *Neospora* species. J. Parasitol. 86, 1366–1368.

Soldati, S., Kiupel, M., Wise, A., Maes, R., Botteron, C., Robert, N., 2004. Meningoencephalomyelitis caused by *Neospora caninum* in a juvenile fallow deer (*Dama dama*). J. Vet. Med. A 51, 280–283.

Söndgen, P., Peters, M., Bärwald, A., Wurm, R., Holling, F., Conraths, F.J., Schares, G., 2001. Bovine neosporosis: immunoblot improves foetal serology. Vet. Parasitol. 102, 279–290.

Sreekumar, C., Hill, D.E., Fournet, V.M., Rosenthal, B.M., Lindsay, D.S., Dubey, J.P., 2003. Detection of *Hammondia heydorni*-like organisms and their differentiation from *Neospora caninum* using random-amplified polymorphic DNA-polymerase chain reaction. J. Parasitol. 89, 1082–1085.

Sreekumar, C., Hill, D.E., Miska, K.B., Rosenthal, B.M., Vianna, M.C.B., Venturini, L., Basso, W., Gennari, S.M., Lindsay, D.S., Dubey, J.P., 2004. *Hammondia heydorni*: evidence of genetic diversity among isolates from dogs. Exp. Parasitol. 107, 65–71.

Stenlund, S., Björkman, C., Holmdahl, O.J.M., Kindahl, H., Uggla, A., 1997. Characterisation of a Swedish bovine isolate of *Neospora caninum*. Parasitol. Res. 83, 214–219.

Stenlund, S., Kindahl, H., Magnusson, U., Uggla, A., Björkman, C., 1999. Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*. Vet. Parasitol. 85, 227–234.

Swift, B.L., Kennedy, P.C., 1972. Experimentally induced infection of in utero bovine fetuses with bovine parainfluenza 3 virus. Am. J. Vet. Res. 33, 57–63.

Thornton, R.N., Gajadhar, A., Evans, J., 1994. *Neospora* abortion epidemic in a dairy herd. N.Z. Vet. J. 42, 190–191.

Thurmond, M., Hietala, S., 1995. Strategies to control *Neospora* infection in cattle. Bovine Pract. 29, 60–63.

Thurmond, M.C., Anderson, M.L., Blanchard, P.C., 1995. Secular and seasonal trends of *Neospora* abortion in California dairy cows. J. Parasitol. 81, 364–367.

Thurmond, M.C., Hietala, S.K., 1996. Culling associated with *Neospora caninum* infection in dairy cows. Am. J. Vet. Res. 57, 1559–1562.

Thurmond, M.C., Hietala, S.K., 1997. Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. Am. J. Vet. Res. 58, 1381–1385.

Thurmond, M.C., Hietala, S.K., Blanchard, P.C., 1997. Herd-based diagnosis of *Neospora caninum*-induced endemic and epidemic abortion in cows and evidence for congenital and postnatal transmission. J. Vet. Diagn. Invest. 9, 44–49.

Tiwari, A., VanLeeuwen, J.A., Dohoo, I.R., Stryhn, H., Keefe, G.P., Haddad, J.P., 2005. Effects of seropositivity for bovine leukemia virus, bovine viral diarrhoea virus, *Mycobacterium avium* subspecies paratuberculosis, and *Neospora caninum* on culling in dairy cattle in four Canadian provinces. Vet. Microbiol. 109, 147–158.

Toolan, D.P., 2003. *Neospora caninum* abortion in cattle—a clinical perspective. Irish Vet. J. 56, 404–410.

Trees, A.J., Williams, D.J.L., 2000. Neosporosis in the United Kingdom. Int. J. Parasitol. 30, 891–893.

Trees, A.J., McAllister, M.M., Guy, C.S., McGarry, J.W., Smith, R.F., Williams, D.J.L., 2002. *Neospora caninum*: oocyst challenge of pregnant cows. Vet. Parasitol. 109, 147–154.

Uggla, A., Stenlund, S., Holmdahl, O.J.M., Jakubek, E.-B., Thebo, P., Kindahl, H., Björkman, C., 1998. Oral *Neospora caninum* inoculation of neonatal calves. Int. J. Parasitol. 28, 1467–1472.

Van Maanen, C., Wouda, W., Schares, G., von Blumröder, D., Conraths, F.J., Norton, R., Williams, D.J.L., Esteban-Redondo, I., Innes, E.A., Mattsson, J.G., Björkman, C., Fernández-García, A., Ortega-Mora, L.M., Müller, N., Sager, H., Hemphill, A., 2004. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. Vet. Parasitol. 126, 351–364.

VanLeeuwen, J.A., Keefe, G.P., Tremblay, R., Power, C., Wichtel, J.J., 2001. Seroprevalence of infection with *Mycobacterium avium* subspecies *paratuberculosis*, bovine leukemia virus, and bovine viral diarrhea virus in maritime Canada dairy cattle. Can. Vet. J. 42, 193–198.

Varcasia, A., Capelli, G., Ruiu, A., Ladu, M., Scala, A., Bjorkman, C., 2006. Prevalence of *Neospora caninum* infection in Sardinian dairy farms (Italy) detected by iscom ELISA on tank bulk milk. Parasitol. Res. 98, 264–267.

Venturini, M.C., Venturini, L., Bacigalupe, D., Machuca, M., Echaide, I., Basso, W., Unzaga, J.M., Di Lorenzo, C., Guglielmone, A., Jenkins, M.C., Dubey, J.P., 1999. *Neospora caninum* infections in bovine foetuses and dairy cows with abortions in Argentina. Int. J. Parasitol. 29, 1705–1708.

Vianna, M.C.B., Sreekumar, C., Miska, K.B., Hill, D.E., Dubey, J.P., 2005. Isolation of *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*). Vet. Parasitol. 129, 253–257.

Von Blumröder, D., Schares, G., Norton, R., Williams, D.J.L., Esteban-Redondo, I., Wright, S., Björkman, C., Frössling, J., Risco-Castillo, V., Fernández-García, A., Ortega-Mora, L.M., Sager, H., Hemphill, A., van Maanen, C., Wouda, W., Conraths, F.J., 2004. Comparison and standardisation of serological methods for the diagnosis of *Neospora caninum* infection in bovines. Vet. Parasitol. 120, 11–22.

Vonlaufen, N., Gianinazzi, C., Müller, N., Simon, F., Björkman, C., Jungi, T.W., Leib, S.L., Hemphill, A., 2002. Infection of organotypic slice cultures from rat central nervous tissue with *Neospora caninum*: an alternative approach to study host–parasite interactions. Int. J. Parasitol. 32, 533–542.

Waldner, C.L., Janzen, E.D., Ribble, C.S., 1998. Determination of the association between *Neospora caninum* infection and reproductive performance in beef herds. J. Am. Vet. Med. Assoc. 213, 685–690.

Waldner, C.L., Henderson, J., Wu, J.T.Y., Breker, K., Chow, E.Y.W., 2001. Reproductive performance of a cow-calf herd following a *Neospora caninum*-associated abortion epidemic. Can. Vet. J. 42, 355–360.

Waldner, C.L., 2002. Pre-colostral antibodies to *Neospora caninum* in beef calves following an abortion outbreak and associated fall weaning weights. Bovine Pract. 36, 81–85.

Waldner, C.L., Cunningham, G., Campbell, J.R., 2004. Agreement between three serological tests for *Neospora caninum* in beef cattle. J. Vet. Diagn. Invest. 16, 313–315.

Waldner, C.L., 2005. Serological status for *N. caninum*, bovine viral diarrhea virus, and infectious bovine rhinotracheitis virus at pregnancy testing and reproductive performance in beef herds. Anim. Reprod. Sci. 90, 219–242.

Williams, D.J.L., McGarry, J., Guy, F., Barber, J., Trees, A.J., 1997. Novel ELISA for detection of *Neospora*-specific antibodies in cattle. Vet. Rec. 140, 328–331.

Williams, D.J.L., Davison, H.C., Helmick, B., McGarry, J., Guy, F., Otter, A., Trees, A.J., 1999. Evaluation of a commercial ELISA for detecting serum antibody to *Neospora caninum* in cattle. Vet. Rec. 145, 571–575.

Williams, D.J.L., Guy, C.S., McGarry, J.W., Guy, F., Tasker, L., Smith, R.F., MacEachern, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. Parasitology 121, 347–358.

Williams, D.J.L., Guy, C.S., Smith, R.F., Guy, F., McGarry, J.W., McKay, J.S., Trees, A.J., 2003. First demonstration of protective

immunity against foetopathy in cattle with latent *Neospora caninum* infection. Int. J. Parasitol. 33, 1059–1065.

Wouda, W., Dubey, J.P., Jenkins, M.C., 1997a. Serological diagnosis of bovine fetal neosporosis. J. Parasitol. 83, 545–547.

Wouda, W., Moen, A.R., Visser, I.J.R., van Knapen, F., 1997b. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. J. Vet. Diagn. Invest. 9, 180–185.

Wouda, W., Moen, A.R., Schukken, Y.H., 1998a. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. Theriogenology 49, 1311–1316.

Wouda, W., Brinkhof, J., van Maanen, C., de Gee, A.L.W., Moen, A.R., 1998b. Serodiagnosis of neosporosis in individual cows and dairy herds: a comparative study of three enzyme-linked immunosorbent assays. Clin. Diagn. Lab. Immunol. 5, 711–716.

Wouda, W., Bartels, C.J., Moen, A.R., 1999. Characteristics of *Neospora caninum*-associated abortion storms in dairy herds in the Netherlands (1995–1997). Theriogenology 52, 233–245.

Wu, J.T., Dreger, S., Chow, E.Y., Bowlby, E.E., 2002. Validation of 2 commercial *Neospora caninum* antibody enzyme linked immunosorbent assays. Can. J. Vet. Res. 66, 264–271.

Yaeger, M.J., Shawd-Wessels, S., Leslie-Steen, P., 1994. *Neospora* abortion storm in a midwestern dairy. J. Vet. Diagn. Invest. 6, 506–508.

Yamage, M., Flechtner, O., Gottstein, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally-infected nude mice by the polymerase chain reaction (PCR). J. Parasitol. 82, 272–279.

Yamane, I., Kokuho, T., Shimura, K., Eto, M., Shibahara, T., Haritani, M., Ouchi, Y., Sverlow, K., Conrad, P.A., 1997. In vitro isolation and characterisation of a bovine *Neospora species* in Japan. Res. Vet. Sci. 63, 77–80.

Yamane, I., Shibahara, T., Kokuho, T., Shimura, K., Hamaoka, T., Haritani, M., Conrad, P.A., Park, C.H., Sawada, M., Umemura, T., 1998. An improved isolation technique for bovine *Neospora* species. J. Vet. Diagn. Invest. 10, 364–368.

Zintl, A., Pennington, S.R., Mulcahay, G., 2006. Comparison of different methods for the solubilisation of *Neospora caninum* (Phylum Apicomplexa) antigen. Vet. Parasitol. 135, 205–213.